Volume 24, No. 4

July-August 1959

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A PROCEDURE FOR THE SIMULTANEOUS IDENTIFICATION OF THE N-TERMINAL RESIDUES AND THE DETERMINATION OF THE AMINO ACID COMPOSITION OF PROTEINS

N. N. Mel'teva

Department of Chemistry, Fr. Éngels Institute of Soviet Trade, Leningrad

The method for the identification of the N-terminal residues of proteins, depending on treatment with fluorodinitrobenzene, followed by chromatographic separation of the dinitrophenyl derivatives of amino acids (DNP-amino acids), has been extensively applied of recent years to the elucidation of the structure of proteins. On the other hand, chromatographic analysis has provided fairly exhaustive and precise data on the amino acid composition of proteins.

In the present paper we described the results of simultaneous identification of the N-terminal residues of proteins and determination of their amino acid composition, by treating the proteins with fluorodinitrobenzene (FDNB), with subsequent separation of mixtures of amino acids and DNP-amino acids by chromatographic methods.

We examined different classes of proteins: zein (prolamine) and other proteins (glutelin) of corn, edestin (globulin) from hemp seed and oryzenin (glutelin) of rice. As a control protein we used crystalline insulin, which has been exhaustively investigated by a number of authors [1].

EXPERIMENTAL

The usual procedures were applied to the preparation of zein and other proteins (glutelin) from Mapledele corn, of edestin from hemp seed, and of oryzenin from rice.

Preparation of DNP-proteins. We added a solution of 0.1 g of FDNB in 2 ml of alcohol to a mixture of 0.1 g of protein and 0.1 g of $NaHCO_3$. The mixture was shaken at room temperature for two hours. If the protein was alkali-soluble, we added the $NaHCO_3$ as an 8% solution. The mixture was then stirred, allowed to settle, and the supernate was poured off. The residue was washed with alcohol, to remove unreacted FDNB, as well as part of the dinitrophenol and dinitroaniline which had been formed during the reaction, then with water, to remove soda and more of the dinitrophenol, and then again with alcohol and ether, in order to dry the product (DNP-zein cannot be treated with ether, with which it forms a sticky mass). The yield of DNP-protein amounted to 0.12 – 0.15 g.

Hydrolysis of DNP-protein and isolation of ether-soluble DNP-amino acids. The DNP-proteins (0.12-0.15 g portions) were placed in a sealed tube with 6 ml of 20% hydrochloric acid, and the tubes were immersed in a boiling water bath for 6 hours (12 hours in the case of DNP-insulin), after which the contents of the tubes were filtered to remove humins, and the filtrates were extracted with ether. The ethereal extracts were dried by the addition of a small amount of calcium chloride, and the ether was cautiously evaporated off. The residues were dissolved in 1 ml of ethyl acetate, and the solutions were taken for chromatographic analysis. It is very important that the DNP derivatives should be shielded from daylight.

Separation of water-soluble DNP-amino acids. After ether extraction, the acid hydrolyzates, which were colored bright yellow if they contained mono-DNP-lysine, were extracted with water-saturated butanol until all

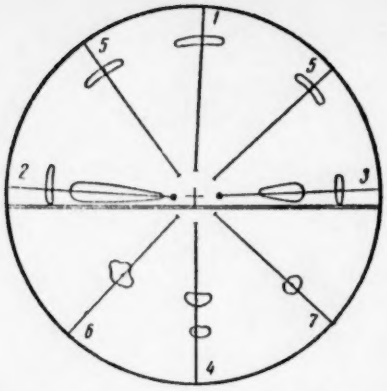

Fig. 1. Chromatogram of the ether-soluble fraction of DNP-protein hydrolyzates. Solvent: sodium phosphate, pH 6.2. DNP derivatives of: 1) insulin; 2) edestin; 3) oryzenin; 4) other corn proteins; 5) glycine; 6) dinitroaniline; 7) dinitrophenol

the color had been removed. The butanol was evaporated off at 25-30°, and the residue was dissolved in 1 ml of ethanol. The acid solution remaining after the butanol extraction was allowed to stand at room temperature until all traces of butanol had evaporated off, and was then placed in a 1 ml pycnometer.

Identification of N-terminal Residues of Proteins

Chromatographic analysis of ether-soluble DNP derivatives

Four different solvents were used for the analysis of the ether-soluble DNP-amino acids: 1) aqueous sodium citrate (pH 6.2) and sodium phosphate buffers (pH 6.2, pH 5, and pH 7); 2) water-saturated phenol; 3) "toluene" (toluene – pyridine – ethylenechlorohydrin – water, 1:1:0.1:0.1 by volume); 4) butanol – acetic acid – water (4 : 5 : 1 volumes).

Circular chromatograms were prepared. Circles of 30 cm diameter were cut out of chromatography paper, and were divided into 8 sectors; the spots were applied on the lines marking the boundaries of the sectors, at a distance of 1.5 cm from the center point. A filter-paper wick was inserted through the center of the circle, dipping into the solvent, which was in a flask placed in a desiccator. The length of the wick was such that it projected 0.5 cm above the level of the paper, which rested on the lip of the desiccator. Water was placed in the lower part of the desiccator, and the lid was placed in position. The results are shown in Table 1 and by the chromatograms of Figs. 1 and 2.

As is shown by the results presented in Table 1, the N-terminal residues of zein prepared from Mapledele corn are threonine and glutamic acid. We were not able to identify the N-terminal amino acids of corn protein other than zein by the dinitrophenyl derivative method. It is evident from Fig. 1 that the two spots given by the ether-soluble fraction of the hydrolyzate from the DNP derivative of this protein fraction were parasitic spots due to dinitrophenol and dinitroaniline. The N-terminal amino acid of oryzenin prepared from commercial rice was glygine, and that of edestin from commercial hemp seed was also glycine.

Chromatographic analysis of water-soluble DNP-amino acids

Butanol extraction of the yellow aqueous hydrolyzate of DNP-proteins removes not only water-soluble DNP-amino acids, but also to some extent other amino acids released as a result of the hydrolysis of the protein. When the chromatograms are subsequently sprayed with ninhydrin, the color of the spots due to mono-DNP-lysine and to N-DNP-histidine changes, and spots due to unsubstituted amino acids also appear; this makes it difficult to identify the spots. So as to overcome this difficulty we developed the chromatograms of water-soluble DNP-amino acids with the solvent butanol – acetic acid – water (4 : 5 :1), the chief merit of which was that the bulk of the amino acid spots were grouped near the starting point, the fastest running one, due to leucine, having a R_f value not exceeding 0.66, while the yellow spots due to DNP-amino acids, was grouped about halfway to the solvent front.

The results of the experiments are presented in Table 2. This table shows that with sufficiently long chromatograms of the butanol-soluble fractions of DNP-protein hydrolyzates, four spots are given by hydrolyzates of DNP derivatives of corn proteins other than zein, of oryzenin, and of edestin, while DNP-zein hydrolyzate gives three yellow spots.

The first spot (R_f 0.53-0.58) corresponds to that given by N-DNP-histidine, which we synthesized by Sanger's method [2]. We were not, however, able to prepare it in a pure state: the pale yellow spot given by N-DNP-histidine, turning brown after ninhydrin spraying (R_f 0.58) was accompanied by a yellow spot due to bis-DNP-histidine (R_f 0.92), giving the blue coloration of free histidine (R_f 0.1) after ninhydrin spraying.

Mono-DNP-lysine (R_f 0.64-0.78) moves together with the second spot given by DNP-protein hydrolyzate (R_f 0.66-0.78), which turns brown after spraying with nindydrin. Since the R_f values of these spots are very

TABLE 1

R_f Values of Ether-Soluble DNP Derivatives

Solvent	Spots given by DNP- protein hydrolyzates																DNP derivatives of				Dinitro-phenol	Dinitro-aniline
	insulin			edestin			oryzenin			Corn protein other than zein			zein				gly-cine	phen-ylala-nine	thre-onine	glu-tamic acid		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III							
Phosphate buffer (pH 6.2)		0.78		0.35	0.78		0.41	0.67		0.47		0.65	0.40	0.59	0.79	0.70		0.81		0.60	
		0.76		0.43	0.76		0.40	0.74		0.48		0.66	0.40	0.60	0.79	0.79	0.78		0.82	0.66	0.49
"Toluene"																						
		0.60	0.85	0.59	0.70	0.96	0.55	0.74	0.97	0.70		0.97	0.30	0.52	0.95	0.51	0.83	0.50	0.31	0.69	0.97
							0.59	0.71	0.97	0.64		0.98	0.58	0.69	0.85	0.58	0.94	0.86	0.60	0.70	0.98
Phenol	0.70	0.84	0.94	0.62	0.78		0.65	0.78		0.64		0.98				0.80				0.66	0.98
								0.80	0.94			0.95				0.83					
Butanol—acetic acid		0.82	0.97					0.79	0.95			0.97				0.82	0.96			0.95	0.97

Note: The R_f values shown in the Table are generally the means of a number of separate experiments. The values enclosed in boxes refer to the R_f values for the spots shown in the chromatograms of Figs. 1 and 2.

variable, and since identification of lysine among the hydrolysis products is of considerable importance, in particular for the corn proteins other than zein, we cut out the spot of R_f 0.64-0.78, and extracted it with 0.1% $NaHCO_3$, with ammonia treatment as recommended by Lowther [3].

The chromatograms of the sodium bicarbonate extracts showed two spots, after spraying with ninhydrin: a brown spot due to mono-DNP-lysine, and a violet spot due to leucine (R_f 0.66), which was extracted together with mono-DNP-lysine (R_f 0.63-0.77). When the bicarbonate extract was treated with ammonia, and chromatographed under identical conditions, four spots were obtained: a violet spot due to lysine formed from mono-DNP-lysine; a yellow spot due to unreacted mono-DNP-lysine; a violet spot given by leucine; and a yellow spot given by dinitrophenol, formed by the action of ammonia on mono-DNP-lysine (Fig. 3).

The third spot given by DNP-protein hydrolyzates (R_f 0.80) was very faint, and we were not able to identify it. It appears, on the basis of published data, to be α-N^1-DNP-arginine (R_f 0.81), which is known not to change color when treated with ninhydrin [4].

The fourth spot (R_f 0.92) moved close to the solvent front, and appears to be due to dinitrophenol (R_f 0.92) together with dinitroaniline (R_f 0.95). Its intensity of coloration fell somewhat after treatment with hydrochloric acid.

We have thus identified mono-DNP-lysine and N-DNP-histidine as being present in butanol extracts of hydrolyzates of dinitrophenyl derivatives of oryzenin, edestin, and corn proteins other than zein. The butanol extract of DNP-zein hydrolyzate contained N-DNP-histidine.

Determination of the amino acid composition of proteins.

The amino acids present in the hydrolyzate after butanol extraction were identified by means of two-way chromatographic analysis.

We used chromatography paper manufactured by the Volodarskii Paper Factory. Two solvent systems were used in succession for development of the chromatograms. The first was a pyridine – acetic acid – water

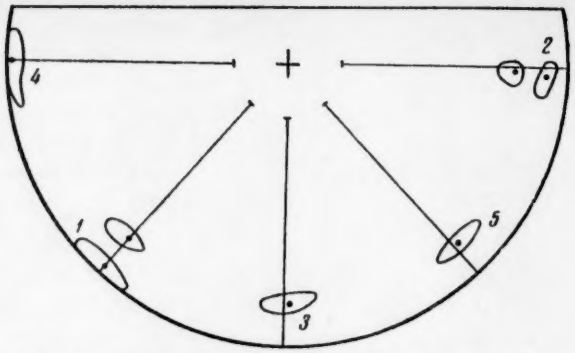

Fig. 2. Chromatogram of the ether-soluble fraction of DNP-protein hydrolyzates. Solvent: butanol – acetic acid – water. DNP derivatives of: 1) insulin; 2) oryzenin; 3) glycine; 4) phenylalanine; 5) dinitrophenol

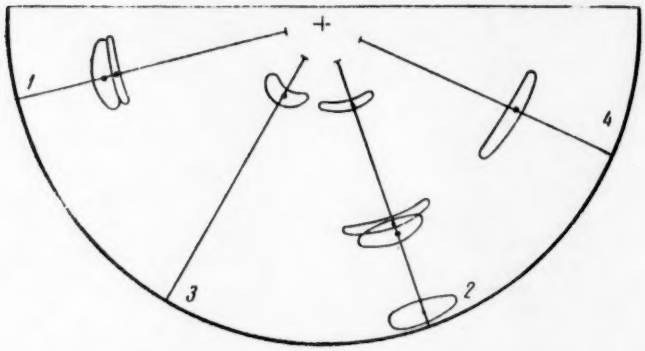

Fig. 3. Chromatogram of a sodium bicarbonate extract of the mono-DNP-lysine spot, sprayed with 0.2% ninhydrin. Solvent: butanol – acetic acid – water. 1) sodium bicarbonate extract, before treatment with ammonia; 2) sodium bicarbonate extract after ammonia treatment; 3) lysine; 4) leucine

(15 : 5 : 3) mixture, and the second either butanol – acetic acid – water (4 : 1 : 5) or water-saturated phenol. After drying, the chromatograms were sprayed with 0.2% ninhydrin in butanol. For the better separation of the spots, we varied the times of development of the chromatograms.

The development time taken for chromatograms of the dimensions 35 x 27.5 cm (the width of half a standard sheet of chromatography paper) was from seven to eight hours. For separation of the complex of leucines, or of threonine from glutamic acid, the time of development was prolonged to 24-30 hours, the solvent being allowed to flow freely off the paper. As a control, we took a DNP-insulin hydrolyzate which had been extracted successively with ether and butanol (Fig. 4). In those cases in which there was some uncertainty about the location of a given amino acid, a solution of authentic amino acid was added to the hydrolyzate, which was again chromatographed, and the enhanced intensity of the spot would confirm its identification.

The most difficultly separable amino acids were those of groups in which the R_f values were very close to each other: histidine – arginine; threonine – glutamic acid; valine – methionine. Since, when the proteins are treated with FDNB, lysine and histidine, being in the middle of the chain, yield mono-DNP-lysine and N-DNP-histidine, they may be readily identified as DNP-amino acids in the butanol extract (Table 2). Thus, of the

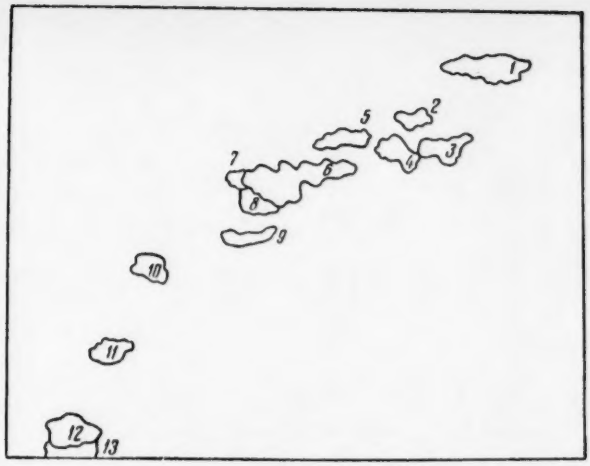

Fig. 4. Chromatogram of the extracted hydrolyzate of DNP-insulin. Solvents: pyridine – acetic acid – water; butanol – acetic acid – water. Amino acids: 1) cystine; 2) arginine; 3) aspartic acid; 4) glycine; 5) serine; 6) glutamic acid; 7) threonine; 8) alanine; 9) proline; 10) tyrosine; 11) valine; 12) phenylalanine; 13) leucine

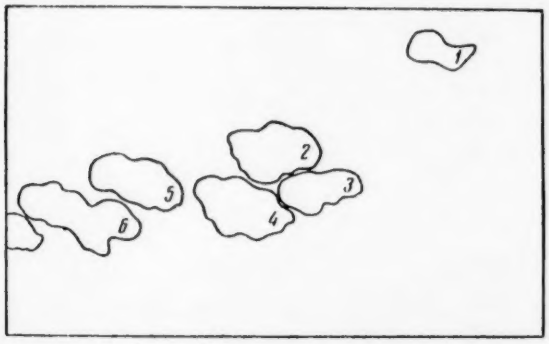

Fig. 5. Upper part of a chromatogram of the aqueous residue after extraction of a hydrolyzate of DNP derivative of corn proteins other than zein. Solvents: pyridine – acetic acid – water; butanol – acetic acid – water. Amino acids: 1) cystine; 2) arginine; 3) aspartic acid; 4) glycine; 5) serine; 6) glutamic acid

Fig. 6. Upper part of a chromatogram of the aqueous residue after extraction of a hydrolyzate of DNP-zein. Solvents: pyridine – acetic acid – water; water-saturated phenol. Amino acids: 1) cystine; 2) aspartic acid; 3) serine; 4) glutamic acid; 5) threonine; 6) alanine; 7) tyrosine

six-carbon amino acids, which are usually difficultly separable from each other, only arginine remains, and this is very readily distinguishable on the chromatograms (Fig. 5).

We were able to separate the threonine and glutamic acid spots, using water-saturated phenol as the solvent (Fig. 6). Since amino acids move at a different rate with phenol than with butanol – acetic acid – water mixture, the spots due to arginine, glycine and proline, which move very fast with phenol solvent, do not appear on this chromatogram, although threonine was clearly separated. We were not able to separate valine from methionine.

We were able in this way to show that the proteins of Mapledele corn, other than zein, contain 16 amino acids: cystine, arginine, aspartic acid, glycine, serine, glutamic acid, threonine, alanine, proline, tyrosine,

TABLE 2

R_f Values of Water-Soluble DNP derivatives (Solvent: Butanol — Acetic Acid — Water)

Distance traveled by the solvent front, in mm	Protein		Spots given by the DNP-protein hydrolyzate				N-DNP-histidine	Mono-DNP-lysine	DNP-leucine	Bis-DNP-histidine	Dinitrophenol	Dinitroaniline
			I	II	III	IV						
225—230 238—240	Zein (12 experiments)		{0.54 {0.54		{0.84 {0.80	{0.96 {0.95	0.58	0.64	0.66	0.92	0.93	0.97
			{0.51 {0.52		{0.78 {0.78	{0.95 {0.92	{(0.10) {(0.92)	0.63				
285—286 320—336	Corn proteins other than zein (90 experiments)	Mean	0.53		0.80	0.95	0.58	0.63	0.66	0.92	0.93	0.97
			{0.57 {0.57	{0.76 {0.79	0.80	0.92		0.78 0.77			0.92	0.96
			{0.58 {0.59	{0.78 {0.77	0.80	0.96		0.77				
		Mean										
280—285	Edestin (30 experiments)	Mean	0.58	0.77	0.80	0.94		0.77			0.92	0.96
			{0.52	{0.65	{0.79	{0.90						
			{0.53	{0.67	{0.80	{0.96		0.66	0.66		0.93	0.95
290—300	Oryzenin (30 expts.)	Mean	0.53	0.66	0.80	0.93		0.66			0.93	0.95
			{0.53	{0.68	{0.80	{0.90						
			{0.54	{0.70	{0.79	{0.95		0.65	0.66			0.97
		Mean	0.53	0.69	0.80	0.93		0.65				0.97

Note: The figures bracketed together refer to R_f values found for parallel chromatograms; each value is usually the mean of three experiments.

valine, methionine, phenylalanine, leucine, lysine and histidine. Tryptophan was identified in a separate portion of protein by means of reactions characteristic of the indole ring.

By the same methods, identifying lysine and histidine as their mono-DNP-derivatives (Table 2), tryptophan in a separate portion of protein, and the remaining amino acids by two-dimensional paper chromatography were able to show that edestin contains the same 17 amino acids, and that Mapledele corn zein contains cystine, arginine, aspartic acid, glycine, serine, glutamic acid, threonine, alanine, proline, tyrosine, valine and methionine, phenylalanine, leucine and histidine. As was expected, zein does not contain lysine and tryptophan.

SUMMARY

A method for the simultaneous identification of the N-terminal amino acids of proteins, and for the determination of the amino acid composition, is described. The method was tested on four different classes of proteins. We used crystalline insulin as the control protein. We identified the N-terminal residues of zein from Mapledele corn, and of edestin and oryzenin prepared from commercial hemp seed and rice. We found that the fraction of Mapledele corn proteins other than zein did not contain N-terminal residues which could be identified as dinitrophenyl derivatives.

The amino acid composition of zein and other proteins of Mapledele corn, and of edestin, was determined qualitatively, identifying lysine and histidine as mono-DNP-derivatives, and the remaining amino acids by means of two-dimensional paper chromatography. All the proteins except zein, which does not contain lysine, contained 16 different amino acids: cystine, argine, aspartic acid, glycine, serine, glutamic acid, threonine, alanine, proline, tyrosine, valine and methionine, phenylalanine, leucine, histidine and lysine.

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NATURE OF THE PROCESS OF DEGRADATION OF PROTEINS BY THE ACTION OF PROTEOLYTIC ENZYMES

V. V. Mosolov

A. N. Bakh Institute of Biochemistry, Academy of Sciences USSR

Considerable advances have been made of recent years in the study of proteolytic enzymes. The processes of activation of a number of proenzymes have been largely elucidated, such as for the cases of trypsinogen, chymotrypsinogen, and pepsinogen, and the specificity of action of a number of proteolytic enzymes has been studied using synthetic substrates and proteins; work is also in progress on the elucidation of the structure of the active site of proteinases. In spite of this, however, many of the problems of the process of proteolysis still remain unsolved; among these is that of the nature of the process of breakdown of the protein molecule under the action of proteolytic enzymes.

Three possible pathways of proteolysis may theoretically be envisaged: a) progressive splitting off from the protein molecule of terminal low-molecular hydrolysis products, leaving a high-molecular residue during the initial stages of this process, and exposing to further attack by the enzyme; b) initial splitting up of the protein molecule into large fragments, which are then gradually degraded to the final products; c) "explosive" breakdown of the protein molecule as a whole, with immediate production of the final reaction products.

The first of these pathways appears to be characteristic of the peptidases, which detach terminal amino acids one by one (carboxypeptidases, leucine aminopeptidase). The second and third pathways apply probably to the action of the true proteinases, although for some of these the first pathways may be possible, as, for example, for the processes of activation of zymogens of proteolytic enzymes.

The object of the present research was to gain further information on the nature of the process of degradation of proteins by the action of proteolytic enzymes, so as to be able subsequently to approach the problem of elucidation of the more general laws regulating the proteolytic process.

For the investigation of proteolysis we used the method of paper electrophoresis; with the use of the appropriate staining procedures, this method gives a representation of all the hydrolysis products at the same time, irrespective of their molecular weights. We took human serum albumin* as the substrate. The enzyme used was crystalline pepsin [1]. Digestion was effected in acetate – HCl buffer at 37°. We measured the pH potentiometrically during the process, and when necessary we adjusted the pH to the requisite value by adding N HCl. Electrophoresis was conducted in a horizontal tank, with water-cooling, at a potential gradient of 20-22 v/cm, in borate buffer of pH 8.4, μ = 0.17, strength of current 0.4 ma/cm. Ten μl portions of hydrolyzate (corresponding to 600-700 μg of the original protein) were applied to damp strips of slow chromatographic paper, the dimensions of the strips being 3 × 52 cm. After completing electrophoresis, we placed the strips in a drying oven at 110° for 10 min , and then stained them with 0.1% bromophenol blue (BPB) in 96% ethanol saturated with mercuric chloride [2]. Excess dye was washed off with tap water. The washed, dried electrophoretograms were analyzed in a Shipalov densitometer [3]. The content of each electrophoretic fraction was calculated from the area under the corresponding peak of the densitometer curves. We also tried staining the electrophoretograms

*The albumin was supplied by the Moscow I. I. Mechnikov Institute of Vaccines and Sera.

TABLE 1

Nonprotein and Amino Nitrogen Contents During the Course of Peptic Digestion of Serum Albumin at pH 1.5*

Time of hydrolysis	Nonprotein nitrogen as % of total nitrogen		Amino nitrogen, as % of total nitrogen	
	E:S - 1:450	E:S - 1:50	E:S - 1:450	E:S - 1:50
5 min	—	29.1	—	6.00
15 »	28.4	46.5	5.02	7.05
30 »	38.8	70.9	—	7.78
1 hr.	50.5	96.8	6.78	7.86
3 »	72.9	98.5	7.82	8.70
24 »	89.4	99.8	11.24	10.50
48 »	95.4	—	12.32	—
72 »	98.4	—	12.79	—

* The analyses for nonprotein nitrogen were performed by Yu. P. Mel'nichuk, a graduate student of the M. V. Lomonosov Moscow State University.

with Penceau 2R [4], but we found that this dye was unsuitable, as some of the proteolysis products remained unstained. Treatment with ninhydrin revealed the same components as did staining with BPB, but the intensity of coloration given by ninhydrin was much less. Thus, staining with BPB was found to give the best results with the proteolysis products formed under our experimental conditions. This finding was not unexpected, since it is known that even such comparatively low-molecular weight peptides as oxytocin and vasopressin are readily stained with BPB [5]. Nonprotein nitrogen was determined in the filtrates from precipitation of proteins by adding trichloroacetic acid to a final concentration of 5%. Amino nitrogen was determined by formol titration. Total nitrogen was determined by a macro-Kjeldahl method.

Fig. 1 shows a series of electrophoretograms, illustrating the process of hydrolysis of serum albumin by pepsin at pH 1.5, with an enzyme – substrate ratio (E : S) of 1 : 450. Comparison of the electrophoretograms of samples taken at different times after beginning digestion shows that the process of enzymatic hydrolysis of protein proceeds through a series of intermediate degradation products which undergo further degradation as digestion progresses. The original protein was electrophoretically homogeneous under our experimental conditions, moving towards the anode. For comparison with the electrophoresis data we present in Table 1, the results of determinations of nonprotein and amino nitrogen at various stages of digestion of albumin, at two different enzyme – substrate ratios.

Considerable interest attaches to a comparison of the relative rates of formation of nonprotein and amino nitrogen during proteolysis. The "explosive" type of breakdown of the protein molecule, leading to the direct formation of the final products over the whole of the course of the proteolytic reaction, would require that there should be a linear relation between the increments in nonprotein and amino nitrogen [6]. Fig. 2 shows that amino nitrogen continues to rise after formation of nonprotein nitrogen has practically ceased. Our results therefore provide evidence of the gradual nature of breakdown of protein in our experiments. An examination of the electrophoretograms of Fig. 1 shows the presence of a component, marked with the letter B, which makes its appearance at an early stage of proteolysis; its content rises with time up to 24 hr , and is still considerable after 48 hr of hydrolysis. It could be supposed that this component represents an intermediate reaction product which has not had enough time for its further degradation during the duration of our experiment. In order to investigate this possibility, we repeated the experiment, changing the E : S ratio to 1 : 50. As is evident from Fig. 3, the component of mobility corresponding with that of component B also appears here, considerable amounts being formed. Its amount begins to fall only after 48 hr of digestion. We hence conclude that the component (or mixture of components) corresponding with band B is probably a substance (or a mixture of substances) which is only slightly susceptible, if at all, to the action of pepsin. It should, however, be remembered, in assessing the content of component B in the hydrolyzate, that the intensity of coloration of the band on the electrophoretogram is determined in the first place by the affinity of the component for the dye, and is not necessarily proportional to its relative content.

For this reason, we determined the nitrogen content of the various electrophoretic fractions of the hydrolyzate, in order to arrive at the true content of fraction B. We streaked 0.6 ml of hydrolyzate on a wide strip of paper (about 16 cm), and conducted electrophoretic separation of the fractions, under the above-described conditions. The paper was then cut longitudinally into narrow strips, 3 cm apart, two of which were stained as above with BPB, to show the locations of the bands. The corresponding parts of the unstained part of the strip were then cut out, and their nitrogen content was determined.

Table 2 shows the distribution of nitrogen among the various fractions of the hydrolyzate obtained in one

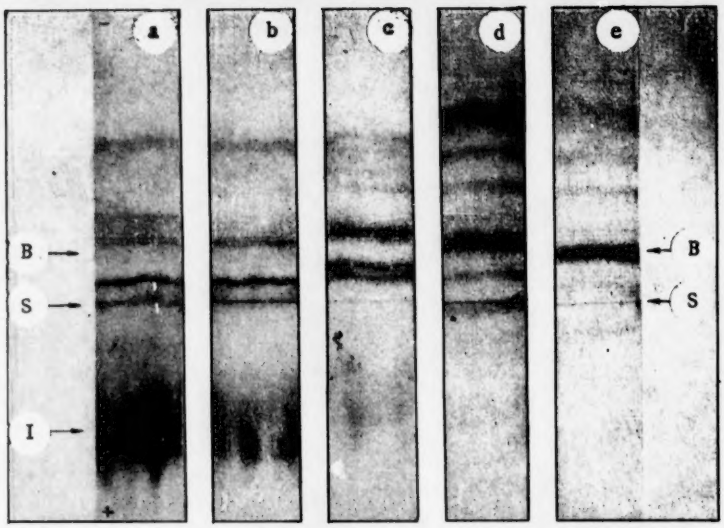

Fig. 1. Peptic hydrolysis of serum albumin: pH 1.5; E : S 1 : 450. Samples were taken at the following times after initiation of proteolysis: a) 30 min ; b) 1 hr ; c) 3 hr ; d) 24 hr ; e) 48 hr ; B — the band due to fraction B; S — starting line; I — initial protein.

TABLE 2

Distribution of Nitrogen Among the Various Fractions of a Peptic Hydrolyzate of Serum Albumin at pH 3.5

Duration of hydrolysis	Nitrogen content of separate fractions					
	E:S =1:450 *			E:S =1:50 **		
	A	B	C	A	B	C
15 min	—	—	—	9.31	13.49	77.16
30 "	2.04	10.27	87.69	—	—	—
1 hr	4.78	8.59	86.63	14.03	16.10	69.86
3 "	4.24	18.64	77.12	20.01	19.00	66.59
24 "	6.75	26.04	67.21	19.65	27.67	52.67
72 "	11.88	36.15	51.97	26.39	18.73	54.87

* The values represent percentages of the sum of the nitrogen contents of all the fractions.

** The values represent percentages of the nitrogen content of the amount of hydrolyzate added to the paper, which was determined separately.

of the experiments. Fraction A represents a substance of high electrophoretic mobility, moving towards the cathode, whereas fraction B represents components moving towards the anode. Undigested protein, which is present during the early stages of digestion, is thus also present in fraction B. The data presented in Table 2 thus confirm the results of visual and densitometric analysis, and they show that the amount of component B rises considerably during the course of peptic digestion of serum albumin, to amount to 20-30% of the total nitrogen content of the protein initially present. Experiments in which the peptic digestion was conducted at high pH values (4.7, 5.3) gave much the same results, although the rate of hydrolysis of protein was much lower.

The results of densitometric estimation of the content of fraction B in the products of digestion at pH 4.6-4.7 are given in Table 3.

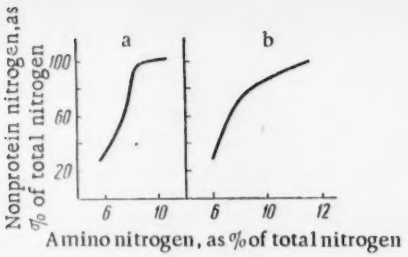

Fig. 2. Relation between amino and nonprotein nitrogen during the course of peptic digestion of serum albumin: pH 1.5; a) E : S = 1:50; b) E : S = 1:450

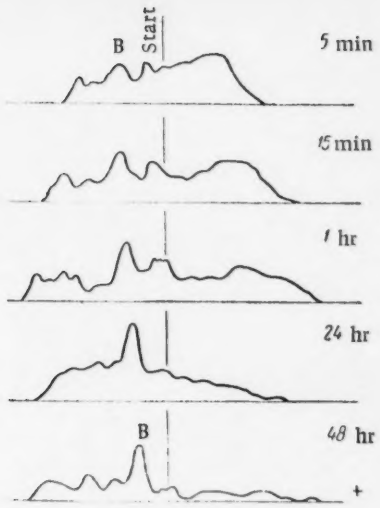

Fig. 3. Peptic hydrolysis of serum albumin: pH 1.5, a) E : S = 1:50 Densitometer recordings [8]

Considerable interest attached to a study of the properties of component B. It was necessary for this purpose to prepare this substance in large amounts. Strips were prepared specially from paper pulp, of a thickness such that large amounts of hydrolyzate could be applied to them. To such strips, 9-10 cm long and about 8 mm thick, we applied 6 ml portions of hydrolyzate (containing the breakdown products of about 300 mg of protein). After completion of electrophoresis, we applied a strip of filter paper to the surface of the thick strip, and stained the imprint with BPB. The segments of the electrophoretogram corresponding with the bands so revealed were thus indicated with sufficient precision. These segments were cut out, and extracted with 5% acetic acid. In order to lower the salt content of the extracted material we extracted it with alcohol, and then dried the alcoholic extract in a vacuum desiccator.

Electrophoretic examination showed that the alcoholic extract contained fraction B, with an admixture of substances of somewhat higher electrophoretic mobility. Densitometer measurements showed that component B accounted for 53-57% of the total fraction staining with BPB. We thought it would be of considerable interest to try to find out whether the high content of component B in the hydrolyzates was due to its resistance to the action of pepsin, or to product inhibition [7], or to the presence in this fraction of specific enzyme inhibitors [8]. With this object, we subjected isolated fraction B to the action of pepsin. Electrophoretograms of fraction B are shown in Fig. 4; (a) immediately after adding pepsin, (b) 1 hour later, (c) 3 hours later, and (d) 24 hours later. It is evident that no appreciable digestion of fraction B by pepsin has taken place. In order to exclude the possibility that fraction B might contain inhibitors of enzyme activity, we added fresh protein to a digest of fraction B after 24 hours of incubation with pepsin, and then incubated for a further 24 hours; the hydrolyzate was then subjected to electrophoresis. It can be seen clearly (Fig. 4,e) that digestion of the added protein had proceeded normally in this experiment (for comparison see Fig. 1, d and Fig. 4, e). It may be concluded from these experiments that the reason for the accumulation of fraction B in the hydrolyzates is that it is resistant to the action of pepsin. Fraction B has a very low electrophoretic mobility, as is shown by comparison with dextran, which is electrically neutral, and which is carried passively along the strip with the flow of solvent [9].

DISCUSSION OF RESULTS

Tiselius and Eriksson-Quensel [10], who in 1939 made a study of the action of pepsin on ovalbumin, came to the conclusion that the protein broke down "explosively" under the action of this enzyme, on the "all or none" principle. In such a case the enzymatic hydrolyzate should be expected to contain intact molecules of the original protein side by side with its final hydrolysis products, but not to contain intermediate degradation products. A number of other authors [6] have arrived at the same conclusions. Yet a considerable number of papers have been published, reporting the appearance during proteolysis of intermediate breakdown products, which differed in their properties from the original proteins, and which were capable of being further degraded by the enzymes in question [11-15]. The authors of these papers concluded that enzymatic attack on the protein molecules was achieved by means of a process of gradual breakdown. Currie and Bull [16] expressed the

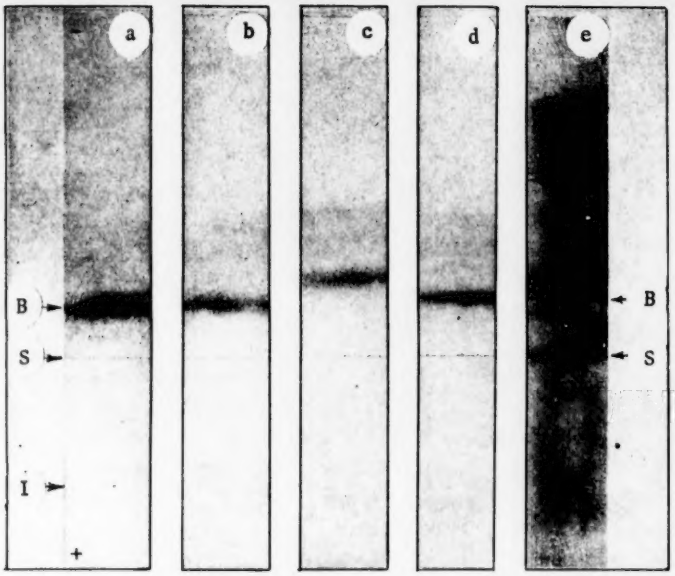

Fig. 4. Peptic digestion of fraction B. For explanation see text.

TABLE 3

Content of Fraction B Determined Densitometrically in a Peptic Hydrolyzate of Serum Albumin at pH 4.6-4.7. The figures represent the areas under the B peaks, as percentages of the whole area stained with BPB

No. of expt.	Duration of hydrolysis				
	0 min	1 hr	3hr	24 hr	48hr
1	8.3	9.0	14.6	21.6	26.6
2	11.5	—	—	20.5	23.4

opinion that the nature of the process of enzymatic proteolysis depends on the experimental conditions, such as pH, temperature, and E:S ratio. Lower temperatures, pH values higher than 2, and higher enzyme concentrations favor the formation of peptides of low molecular weight, at the cost of high molecular weight fragments. Conversely, low pH values, higher temperatures, and lower enzyme concentrations favor the formation of peptides of high molecular weight. It is conceivable that the problem of the apparent nature of the proteolytic process can be reduced to a consideration of the relative rates of breakdown of the initial protein and of the intermediate products. If the rate of breakdown of the initial protein molecules exceeds that of their partial degradation products the process will then appear to be one of stepwise degradation. If, on the contrary, the rate of breakdown of the intermediate degradation products exceeds that of the original protein, then the process will appear to be one of "explosive" breakdown. Substantially the same conclusion was arrived at by Linderstrom-Lang [17], although he identified the initial stage of proteolysis with a process of denaturation. In accordance with the above-expressed view, Chernikov [18] considers that the proteolytic process may be divided into two stages, of which the first is true proteolysis, while the second could be termed "peptidolysis". The ratio of the velocities of these two stages determines untimately the apparent nature of the process of breakdown of the protein molecule.

By the application of paper electrophoresis to the study of the process of digestion of serum albumin by pepsin we were able to show that the protein molecule breaks down in a stepwise manner (Figs. 1 and 3). This is also shown by an examination of the ratios of increase of amino to nonprotein nitrogen during the course of proteolysis (Fig. 2). No radical alterations in the nature of the process were observed when the E:S ratio was varied by a factor of 9, or over a wide range of pH values (from pH 1.5 to pH 5.3). This disagreement between our results and those of Bull [16] may be due to the short incubation times taken by this author in his experiments. In accordance with the above considerations, changes in the relative speeds of sequential proteolytic reactions

taking place when the pH or the temperature were varied, could have been construed as being changes in the general nature of the proteolytic process. We would emphasize that the stepwise course of the process of breakdown of protein molecules, giving rise to a series of successive intermediate products, follows logically from the concept of the globular protein molecule as an integral unit, in which each single opening of a peptide bond should cause perturbation of the energetic equilibrium of the molecule, and should hence lead to the formation of a new, discrete compound [19]. During the course of the peptic digestion of serum albumin we observed the formation of a definite product – fraction B, of which considerable amounts accumulate, representing 20-30% of the total nitrogen content of the original protein. This observation is of considerable intrinsic interest, the more so that this product is scarcely, if at all, attacked by pepsin. The action of proteolytic enzymes on insulin [20] and growth hormone [21] has been found to involve formation of similar difficultly hydrolyzable products. Similarly, Bull [16] observed formation of peptides of an average molecular weight of 30,000 during the process of peptic hydrolysis of ovalbumin. As has been shown by the results of our experiments, the stability of such products cannot be ascribed to the presence of substances which specifically inhibit the action of the enzymes concerned, or to product inhibition. It is, in this connection of interest that the product accumulating in our digests was of low electrophoretic mobility. Taking into consideration that electrostatic forces acting between enzyme and substrate molecules may play a significant part in enzymatic reactions, it might be thought that the stability of such products might to some extent be due to hindrances to formation of enzyme – substrate complexes between the molecules of such products and of the enzyme. However, the validity of such an assumption needs further investigation, in particular of the electrophoretic properties of fraction B at different pH values. It is of interest that an analogous product, which was electrophoretically neutral at pH 8.6, has been reported as being formed during the peptic digestion of catalase; this product retained enzymatic activity [22].

SUMMARY

The enzymatic breakdown of human serum albumin during digestion with pepsin proceeds in a stepwise manner, the nature of which is not affected by varying the enzyme – substrate ratio from 1:450 to 1:50, or by varying the pH over the range 1.5-5.3. A product which is resistant to the action of pepsin accumulates during the course of digestion of serum albumin. This product has no specific inhibitory effect on the activity of pepsin, and it is of low electrophoretic mobility.

In conclusion, I wish to express my profound gratitude to P. V. Afanas'ev, Doctor of Biological Sciences, for the constant interest shown by him during the prosecution of this research, and for his valuable advice and suggestions.

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**In Russian

EFFECT OF IONIZING RADIATION ON THE PROTEIN METABOLISM OF FISH

Ya. A. Épshtein and N. F. Lavroskaya

Radiobiological Laboratory, All-Union Scientific Research Institute of Lake and River Fish Husbandry, Leningrad

Radioactive isotopes, which may be present as contaminants in the waters of reservoirs, may be accumulated by fish, and retained by them for long periods of time [1-5]. Some importance hence attaches to the study of the sensitivity of fish to radiation, and of the effects of radiation on them. The present research is devoted to these subjects; its accomplishment was made possible thanks to the facilities kindly placed at our disposal in the Biophysics Laboratory of the Urals Branch of the Academy of Sciences.*

METHODS

Our experimental material consisted of crucians (Carassius carassius) caught in Lake Topkoe (Urals). The fish taken for the experiments were of about the same weight (14-18 g), and had been kept for a long time in an aquarium. The volume of water per individual fish amounted to about 1 liter. The water was changed regularly. The fish were fed on wheat groats.

The fish were irradiated in two ways: 1) by a single exposure to γ-radiation from Co^{60}, at dosage levels of 250, 1000, 2000, 4000, 6000, 8000 and 16,000 r. In one case a total of 4000 r was spaced over 4 days, at the rate of 1000 r daily. 2) The fish were kept for various times in a steam of water contaminated with Fe^{59} and Sr^{90}. In some of these experiments sodium ethylenediaminetetra-acetate (EDTA) was added to the water.

Blood taken from 10-12 individuals was pooled for the analyses. The fish were bled by cutting off the tail, and the freely flowing blood was collected in a centrifuge tube. The analyses were performed immediately after collection of the serum.

The serum protein content was determined by Conway's method, after digestion with concentrated H_2SO_4. Fractionation of serum proteins was effected by paper electrophoresis, at a potential gradient of about 10v/cm and a strength of current of 0.7 – 1 ma/cm, in veronal buffer of pH 8.6, ionic strength μ = 0.075. The electrophoretograms were dried at 120-130° for 15-20 min, and were stained for 6 hr with bromophenol blue (bromophenol blue – 0.1 g, $ZnSO_4$, H_2O 31 g, 50% ethyl alcohol 50 ml; the solution was made up to 1 liter with 5% acetic acid, according to the Spinco formula). Excess of dye was washed out with 5% acetic acid, followed by treatment with 10% acetic acid containing 9 g of sodium acetate per liter. The stained bands were cut out of the electrophoretograms, and the stain was extracted from them by alkaline solution. The amount of dye extracted from each band was estimated on a FÉK-M photoelectrocolorimeter, and the protein content of each fraction was hence calculated.

The nonprotein nitrogen content of the serum was determined by Kjeldahl digestion of the trichloroacetic acid filtrate, followed by ammonia determination by Conway's microdiffusion method. The amino nitrogen

* We wish to express our gratitude to the Head of the Laboratory, N. V. Timofeev-Resovskii, and to his staff, for providing us with the laboratory facilities for carrying out this research.

TABLE 1

Dependence of Survival Time on the Dose of γ-Radiation Received by Fish From a Co^{60} Source

Radiation dose, r	Number of fish irradiated	Daily mortality rate following irradiation											
		0	1	2	3	4	5	6	7	12	22	23	24
2-0	10	1	0	0	0	0	0	0	0	0	0	0	0
1000	10	0	0	0	0	0	0	0	0	0	0	0	0
4000	7	0	0	0	0	0	0	3	1	0	0	2	1
4000	12	0	0	0	0	0	0	4	1	—	—	—	—
4×1000	12	0	0	3	0	2	0	0	0	1	0	0	0
8000	11	0	1	6	1	0	0	0	0	0	0	2	1
16 000	12	0	0	5	4	0	0	0	0	0	1	2	0

TABLE 2

Amino Nitrogen Content of the Blood Serum of Crucians Following Exposure to an External Co^{60} Source of γ-Rays

Radiation dose, r	Time after exposure	Amino nitrogen, mg%
1000	1 month	3 9
2000	3 days	6 1
4000	1 hour	5.6
4000	3 days	6.2
4000	3 "	4.5
4×1000	3 "	5.3
4000*	8 days	5.1
6000	1 hour	5.0
6000	3 days	5.6
6000	8 days	4.8
8000	3 days	8.3
8000	3 "	7.3
Control		4,0

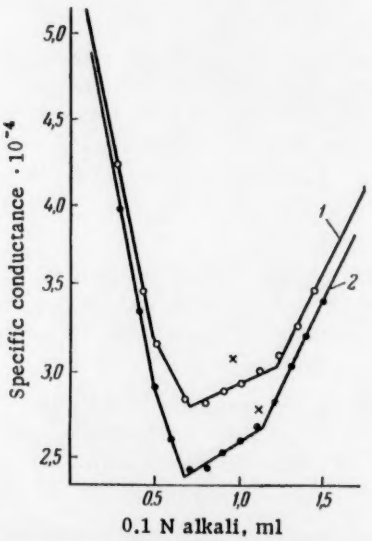

Fig. 1. Conductometric titration curves found for eluates obtained by passing water through columns containing phenolsulfonic resin (in the H form) on which serum from control (2) and irradiated (1) crucians had been sorbed. The + mark indicates the point at which a red coloration, due to phenolphthalein added to the solution, appeared. The serum was taken 3 days after irradiation with a dose of 6000 r.

content of the serum was determined by Uzbekov's method [6].

Acid – base relations in the serum were examined by direct conductometric titration of aqueous eluates obtained by passing water through a phenolsulfonic acid cationite resin (F-2), after addition of 0.1 ml of serum to the resin in the H form [7].

The extent to which serum proteins were denatured by radiation was estimated from the rate of their digestion by pepsin. Since we did not possess the facilities for preparation of pure serum albumin, we subjected the serum as a whole to peptic digestion. We added a 0.3% solution of pepsin in 0.01 N H_2SO_4 to the fresh serum, or to serum after incubation at 29 ± 0.5° for 2.4 hr or more.

The amount of peptolysis was assessed by conductometric titration of 0.1-0.2 ml portions of peptolyzate (corresponding to 0.05-0.1 ml of blood serum), by the method described in an earlier paper[8]. Conductance

TABLE 3

Contents of Chlorides, Buffer Acids* and Groups Titratable in Alkaline Media in Serum Eluates of Control Fish and of Fish Exposed to Radiation from Co^{60}

Radiation dose, r	Time after exposure	Cl	Buffer acids	Titratable alkaline groups
		meq/liter		
1000	1 month	70	36	0
4000	1 hour	70	48	0
4000	3 days	79	31	23
4×1000	3 "	75	48	37
4000	8 "	87	33	19
4000	18 "	65	53	0
6000	1 hour	72	38	0
6000	3 days	73	27	23
6000	11 days	67	44	0
8000	3 days	72	28	10
Control		68.0±2.5	45.8±4.4	Not present

*Not including carbonic acid. The carbonic acid content can be calculated as the difference between the content of all the acids present in serum (150 meq/liter, on the average), and the content of titratable acids (chlorides + buffer acids) [7].

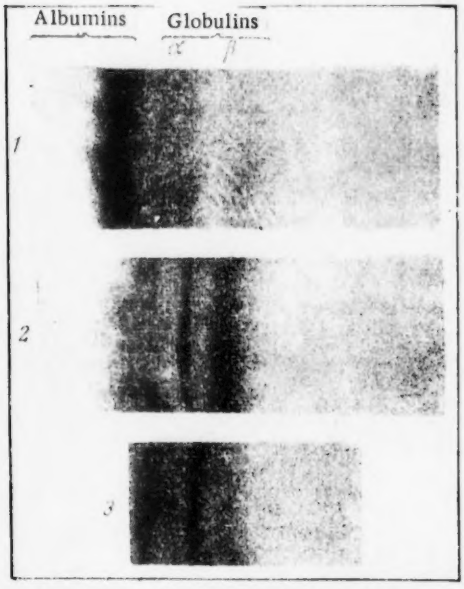

Fig. 2. Paper electrophoretograms of serum proteins: 1) control (rainbow trout); 2) crucian, on the third day after exposure to a dose of 6000 r; 3) crucian, after being kept for 8 days in water containing Fe^{59} (6 μC).

was measured in an instrument for measurement of conductivity and capacity, using a bell-shaped electrode, with a constant of 0.64.

EXPERIMENTAL RESULTS

The survival times of crucians irradiated with γ-rays from a Co^{60} source, at various dosage levels, are given in Table 1.

The death of one of the fish on the day of exposure to a dose of 250 r cannot be attributed to radiation. All the other fish of this group, as well as of that given a dose of 1000 r, survived to the end of the period of observation (48 days), and displayed no differences in behavior from the animals of the control group. Two thirds of the group receiving a dosage of 400 r died, the majority (about half of the entire number) within the first week of exposure. Doses of 8000 and 16,000 r were in all cases lethal, mortality being highest on the 2-3 days after exposure. The dose of radiation causing death of 50% of the fish within 30 days of exposure ($LD_{50/30}$) appears to be about ten times higher than for homeotherms [9], and would be somewhat less than 4000 r.

As has been found to be the case for homeotherms [10], periods of exceptionally high mortality could be distinguished for the fish. Three such mortality peaks

were found for the irradiated crucians: at medium dosage levels mortality was maximum at the end of a week after exposure, while for higher doses the maximum was during the first 2-3 days after exposure; at both dosage levels a third peak was observed at the end of the third and the beginning of the fourth week after irradiation.

It is of interest to compare these findings with those of Aleksandrov [11], who found that mice given a dose of 1500 r died on the third to fourth day after exposure, and with those of Luchnik [10], who distinguished no less than five separate mortality peaks in mice after irradiation.

The amino nitrogen content of the blood serum was found to vary parallel with the dose of radiation received (Table 2).

The following results were obtained for the determination of the amino nitrogen content of the serum of fish kept in water contaminated with radioactive isotopes:

Isotope	Isotope content of the water μC/liter	Duration of exposure, days	Amino nitrogen mg%
Fe^{59}	2.3	14	3.9
	6.0	8	4.7
Sr^{90} + EDTA	2-3	35	3.1
	2-3	35	3.9

TABLE 4

Contents of Chlorides Buffer Acids* and Groups Titratable in Alkaline Media in the Serum of Fish Maintained in Water Contaminated with Radioactive Isotopes

Isotope	Isotope content of the water, μc/liter	Duration of exposure, days	Cl	Buffer acids	Titratable basic groups
			in meq/liter		
Fe^{59}	2,3	14	78	32	16
	6,0	8	64	36	19
Sr^{90}+EDTA	2–3	35	79	42	0
	2–3	35	80	32	0

*Other than carbonic acid

TABLE 5

Fractional Composition of the Serum Proteins of Crucians Kept in Water Contaminated with Radioactive Isotopes

Isotope	Isotope content of the water, μc/liter	Duration of exposure, days	Albumins	Globulins			Albumin/globulin ratio
				α	β	γ	
Fe^{59}	2—3	14	41	29	30	—	0.70
	6	8	41	35	24	—	0.70
	6	8	39	35	26	—	0.64
Sr^{90}+EDTA	2—3	35	39	29	18	13	0.67
	2—3	35	35	30	22	14	0.51

The results of direct titration of eluates of sorbed serum from irradiated fish, and the appearance in these eluates of groups titratable in alkaline media, are shown in Fig. 1 and Table 3. This method permitted the simultaneous determination of the contents of chlorides and of buffer acids other than carbonic acid.

As is shown by the data of Table 3, irradiation from an external Co^{60} source, and irradiation incurred during maintenance in water containing Fe^{59} (Table 4), also constituting intense γ-irradiation, both caused appreciable lowering of the content of water-soluble buffer acids of serum. The content of buffer acids fell to a minimum by about the third day after exposure, and then returned fairly quickly (within 10-18 days) to the normal value.

Substances titratable in alkaline solution make their appearance in the blood serum at a certain definite time after exposure to radiation, and these substances disappear subsequently (Tables 3 and 4).

The total protein content of the serum of irradiated fish varied within the range 1-2%. We could find no correlation between the protein content and the severity of radiation sickness.

TABLE 6

Fractional Composition of the Serum Proteins of Crucians Exposed to γ-Irradiation From an External Co^{60} Source

Radiation dose ρ	Time after exposure	Serum protein fractions, %				Albumin/globulin ratio
		albumins	globulins			
			α	β	γ	
1000	1 month	45	26	30	—	0.80
2000	3 days	45	30	24	—	0.83
4000	1 hour	44	49	29	—	0.76
4000	3 days	49	21	22	11	0.90
4000	3 "	47	28	25	—	0.89
4×1000	3 "	45	27	29	—	0.80
4000	7 "	44	26	31	—	0.77
4000	45 "	47	28	25	—	0.89
6000	1 hour	41	25	33	—	0.71
6000	3 days	50	20	30	—	1.00
6000	11 days	43	26	31	—	0.75
8000	3 "	48	29	23	—	0.92
8000	3 "	50	28	23	—	1.00
Control	Crucian	43±1.4	30±1.4	26±1.5	—	0.77
	Rainbow trout	44.2±2.0	32.0±1	22.4±1.3	—	0.66

The number of protein fractions appearing on the electrophoretograms of the serum of unirradiated crucians and other fish (trout, carp) is considerably smaller than for homeotherms (Table 5). We could usually distinguish only three well-defined bands, due to albumins and to α- and β-globulins (Fig. 2). An additional band appeared on some of the electrophoretograms; its mobility was close to that of the α_1-globulin fraction of the serum of homeotherms. In most cases the band due to this fraction merged with the albumin band.

Hamoir [12] reported the total or nearly total absence of γ-globulins from the blood serum of fishes. Drilhon [13] found that the γ-globulin band of electrophoretograms of carp serum was usually very faint. Sorvachev was, however, able to distinguish 7-8 protein fractions in paper electrophoretograms of crucian or carp serum [14].

Four protein fractions could be distinguished in the serum of crucians kept for long periods of time in water contaminated with Sr^{90}, as was shown by the paper electrophoretograms (Table 5). The fraction having the electrophoretic mobility of γ-globulin, which is absent from the serum of unirradiated fish, may also resemble mammalian γ-globulin in other respects. Additional experiments would be needed to elucidate this question.

An analysis of the fractional composition of the serum proteins of irradiated fish (Table 6) shows that the

value of the albumin/globulin ratio frequently exceeds the normal figure. This effect may, however, be due not so much to a rise in the relative albumin content as to an increase in the α_1-globulin fraction, which, as has been pointed out, frequently merges with the albumin band.

A relative increase in the β-globulin fraction was also observed in a number of cases, most often in the early stages of radiation sickness, and most clearly expressed after exposure to high doses of radiation.

It is known that denaturation of proteins can be detected from the increased rate of their enzymatic proteolysis [15-17]. The rate of digestion of serum albumin of dogs and other animals rises after irradiation, both in vitro and in vivo [8], evidently as a result of its denaturation.

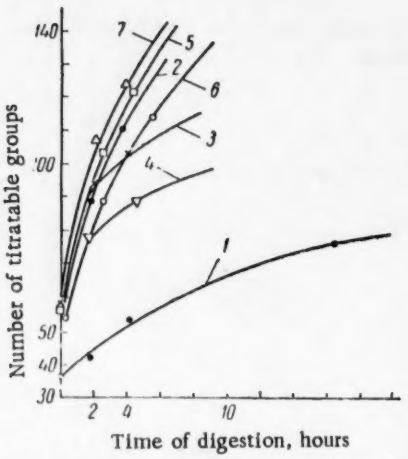

Fig. 3. Curves expressing rates of peptolysis of serum proteins of control (1) and irradiated crucians at different dosage levels (2-7) Times after exposure to a dose of 4000 r : 2) 3 days; 3) 8 days; 4) 17 days. After exposure to 6000 r: 5) 3 days; 6) 11 days. After exposure to 8000 r : 7) 3 days

Fig. 3 illustrates the results of experiments on the rate of peptic digestion of serum proteins of irradiated crucians. The curves show that the rate of digestion rises considerably following irradiation. At the lower dosage levels the rate of proteolysis varied parallel with the amount of radiant energy absorbed. There was very little difference between the effects of doses of 6000 and 8000 r , either of which had little more effect than did doses of 4000 r. It seems probable that it is the circulating proteins which are affected by radiation. This is suggested by the observation that accelerated peptic digestion of the proteins was found one hour after exposure to doses of radiation of 4000 r. In one experiment, however, we could find no change in the rate of peptic digestion of serum proteins one hour after exposure to a dose of 6000 r. In this particular case we were also unable to detect any of the other signs of derangement of protein metabolism, characteristic of radiation sickness.

DISCUSSION

The rise in amino nitrogen content of the blood in radiation sickness may be associated with disturbance of protein metabolism, and is due to progressive increase in the free amino acid content of the blood [9, 3]. Evidently, the same applies to the effects found by us in irradiated fish.

This interpretation would also explain in the observed fall in the content of buffer acids in the blood, coinciding with the appearance of basic groups. If this excess of basic groups is ascribable to the amino groups of amino acids, then about the same number of additional carboxylic groups should appear in the blood serum. In distinction to carboxylic groups present in proteins (which have a pK of about 4), which possess buffer properties, the pK of the carboxylic groups of free amino acids is about 2 [18], i.e., they behave as fairly strong acids, and cannot act as buffer acids. The carboyxlic groups of peptides occupy an intermediate place in this respect. It would thus be expected that, with the same content of basic substances in the serum, the appearance of stronger acids should reduce the content of buffer acids, and the bases with which they were associated would be transferred to the stronger acids.

SUMMARY

Crucians (Carassius carassius), and probably also other fishes, are more resistant to radiation than are homeotherms. The $LD_{50/30}$ was found to be slightly below 4000 r. After exposure to an external Co^{60} source of γ-rays peak mortality was on the second or third day for doses exceeding 4000 r, and at the end of the first week for smaller doses. Shortly after exposure to high doses of radiation, changes appear in the properties of serum proteins, the rate of peptic digestion of which rises. No significant changes could be seen in the total protein content of the serum during a period of four to five weeks following exposure to radiation.

The albumin/globulin ratio of fish subjected to high doses of radiation from Co^{60} tended to rise. This effect may not, however, have been due to an increase in the relative albumin content, but to increase in the content of a globulin fraction, which moves together with the albumin on the electrophoretograms. It was possible in some cases to observe an increase in the relative content of the β-globulin fraction. Similar changes were found in the protein composition of the serum of fish kept in water contaminated with radioactive iron. Such changes were not, however, found when the water was contaminated with Sr^{90}.

The amino nitrogen content of the serum of irradiated fish rises to an extent commensurate with the dose of radiation received. The increase in the content of amino compounds can be detected by direct determination of the acid – base relations of the serum.

With time, all these disturbances of protein metabolism disappear.

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ON THE NATURE AND THE METHODS OF QUANTITATIVE EVALUATION OF THE "HYPERCHROMIC EFFECT" OF NUCLEIC ACIDS

A. S. Spirin, L. P. Gavrilova and A. N. Belozerskii

A. N. Bakh Institute of Biochemistry, Academy of Sciences, USSR, and the Soil Biology Faculty, M. V. Lomonosov State University, Moscow

It has long been known that depolymerization or denaturation of nucleic acids is associated with intensification of their absorption in the ultraviolet (UV). The term "hyperchromic effect" has been given to this phenomenon [1, 2]. Of recent years, experiments on synthetic polynucleotides have shown that the reverse process of formation of polynucleotides from low molecular weight compounds is associated with a fall in UV absorption ("hypochromic effect") [3, 4]. These "effects" must obviously be due to the UV absorption of polynucleotides being lower than that of a mixture of their component nucleotides. This property has been found to apply to the majority of the polynucleotides examined, from the naturally occurring desoxyribonucleic (DNA) and ribonucleic acids (RNA) to the synthetic polyadenylic and polycytidylic and other acids. Inasmuch as the absorption of any of these polynucleotides is always less than the sum of the absorptions of their constituent mononucleotides, it would seem to us to be more reasonable to apply the term "hypochromicity" to this phenomenon, and the term "hypochromic behavior" to the corresponding anomalous behavior of polynucleotides, as compared with mononucleotides.

The causes of this effect have not yet been fully elucidated. It is clear that the fall in UV absorption can be due only to some form of direct interaction between the purine and the pyrimidine residues of the chains. Reactions taking place between other components – carbohydrate or phosphate, and also reactions of phosphorylation or dephosphorylation of the nucleotides, or of esterification of the phosphate residues, do not cause any comparable effect on either the nature or the magnitude of UV absorption of nucleotides [2, 5, 6], and for this reason formation of phosphate diester linkages cannot, by itself, be responsible for the hypochromicity of polynucleotides. In this connection, however, a direct correlation has been shown to exist between the number of hydrogen bonds joining the bases of a pair of DNA chains and the magnitude of the hypochromic effect [2, 7-10]. Rupture of the hydrogen bonds, due to ionization of the basic groups, to thermal denaturation, or to transfer to a medium of low ionic strength, is associated with considerable enhancement of absorption in the UV [7-10], whereas a certain degree of depolymetization involving shortening of the chains, but without rupture of interchain hydrogen bonds appears not to give this effect [10, 11].

There is, however, some evidence that enhancement of UV absorption may also result from the rupture of internucleotide bonds [1, 5, 12-14]. This effect may be ascribed to the smaller mutual reactivity of short polynucleotide chains, due to their greater Brownian motion, and to the less orderly mutual alignment of their molecules. Other explanations cannot, however, be excluded. In particular, there is considerable evidence of the existence of a certain degree of hypochromicity in even such small fragments as the dinucleotides [5-6, 15]. This indicates the possibility of interaction between the planes of the rings of adjacent bases of polynucleotide chains.

The present research represents a contribution to the elucidation of the nature of the hypochromicity of nucleic acids. In addition, we have attempted to provide a simple method for the quantitative evaluation of

the magnitude of the hypochromic effect.

EXPERIMENTAL

Experimental Materials

We examined a number of nucleic acid preparations, and of their derivatives, the preparation and properties of which are described below. Our basic research material was, however, DNA, since its hypochromic behavior and structure have been the most extensively studied.

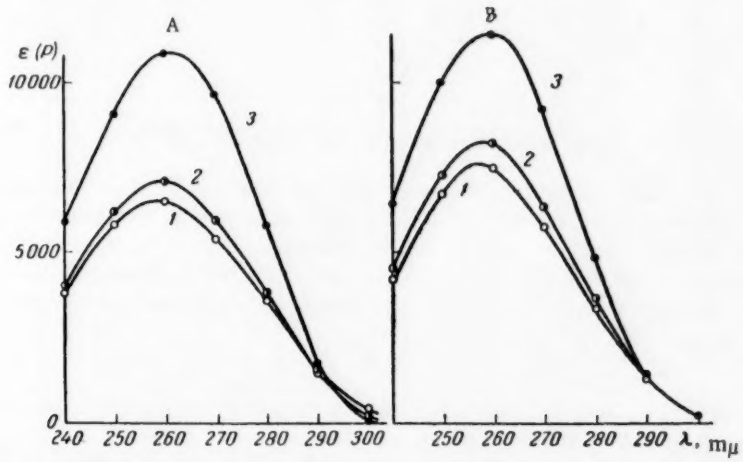

Fig. 1. Absorption curves of nucleic acids and of the corresponding mixtures of mononucleotides (pH 7) A) DNA: 1) native calf thymus DNA; 2) denatured calf thymus DNA, $\mu = 0.1$; 3) calculated theoretically for a mixture of desoxyribonucleotides in the proportions G : A : C : T = 22 : 28 : 22 : 28. B) RNA: 1) native tobacco mosaic virus RNA, $\mu = 1$; 2) native tobacco mosaic virus RNA, $\mu = 0.0005$; 3) solution of mononucleotides obtained by hydrolysis of the RNA with 0.5 N NaOH, 18 hr at 37°

Native DNA. Our preparation of high-polymeric DNA was made from fresh-frozen calf thymus, with preliminary isolation of desoxyribonucleoprotein by Mirsky's method [16], and with subsequent deproteinization according to Kirby [17]. Similarly prepared products from bacteria possessed transforming activity*, which is evidence of the native state of the DNA obtained by these procedures. Our preparation contained not more than 0.5% of protein, as shown by the microbiuret test [18].

Denatured DNA. For the study of fully denatured DNA we took the sodium salt of DNA prepared from calf thymus by Neumann's old method depending on extraction of the material with hot alkali [19]. It has been shown [20] that treatment of DNA with hot alkali totally destroys the original structure of hydrogen bonds, leading to the formation of disordered polynucleotide chains. We subjected the product so obtained to dialysis, which yielded undialyzable desoxyribonucleotides.

Apyrimidinic acids (APyA). Takemura has shown that hydrazinolysis of nucleic acids leads to the selective quantitative elimination from them of the pyrimidine bases, leaving the polynucleotide chain intact [21]. We applied this method to the preparation of APyA from DNA.

One g of DNA sodium salt was heated from 60 min at 60° with 5 ml of anhydrous hydrazine, in a sealed tube. To the cooled product we added four volumes of alcohol, and dissolved the precipitate so formed in

* The biological experiments were performed by D. G. Kudlai, of the N. F. Gamaleya Institute of Microbiology and Epidemiology, AMN SSSR, Moscow.

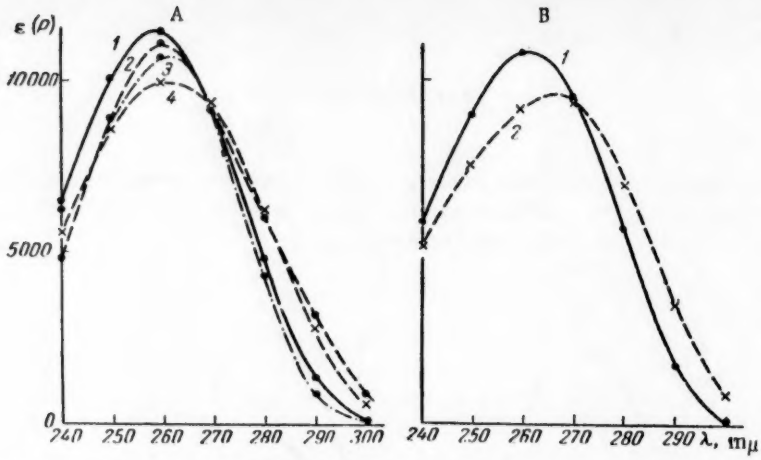

Fig. 2. Absorption curves of nucleic acid hydrolyzates. A) RNA: 1) hydrolyzate obtained by the action of 0.5 N NaOH, for 18 hr at 37° (neutral solution); 2) the same (acid solution); 3) the same (alkaline solution); 4) hydrolyzate obtained by the action of 0.5 N $HClO_4$ at 100°. B) DNA: 1) mixture of free mononucleotides (see Fig. 1) (neutral solution); 2) hydrolyzate obtained by the action of 0.5 N $HClO_4$ at 100°.

water; the solution was made acid to Congo red, and the product was reprecipitated with alcohol. This precipitate was dissolved in phosphate buffer of pH 7.4, and the solution was dialyzed. The resulting precipitate was separated on the centrifuge, and APyA was precipitated from the supernatant by adding alcohol to 20%, with adjustment of the pH to 2-3 (by addition of HCl). The flocculent precipitate was dissolved in phosphate buffer of pH 7.4, and the resulting solution was taken for our experiments. The absence of pyrimidine bases from the product was checked by a spectrophotometric method. The guanine/adenine ratio was found to be close to unity. The APyA thus obtained had the structure of the undialyzed polymer, in which purine desoxyribonucleotides alternated with unsubstituted desoxyribose residues.

Apurinic acid (APuA). According to the findings of Chargaff and his co-workers [22], mild acid hydrolysis of DNA leads to the quantitative elimination of purine bases only, without affecting the polydesoxyribose phosphate chain, or the pyrimidine groups attached to it; the product is APuA. We prepared APuA from our DNA by the method of Tamm and Chargaff [23]. To 50 mg of DNA sodium salt dissolved in 20 ml of water, we added 10 ml of 0.1 N HCl, which brought the pH of the solution to 1.7; a few drops of 0.1 N HCl were added to bring the pH to 1.6, and the turbid solution was dialyzed against 1 liter of dilute HCl (pH 1.6) for 24 hr at 37°. The turbidity disappeared during the process of dialysis. The solution was then exhaustively dialyzed against tap water, and then against a number of changes of 0.15 M NaCl. The resulting solution was used for our experiments.

RNA preparations. For most of our experiments we used native high-molecular RNA from tobacco mosaic virus (TMV), prepared from it by extraction of proteins with phenol, according to Gierer and Schramm [24]. We also used yeast RNA, prepared by extraction with cold solutions of pH 8-9 [19], as well as commercial yeast RNA preparations (German, Hungarian, Merck's).

Experimental Methods

The absorption of any polynucleotide which exhibits hypochromicity is a certain fraction (a certain percentage) of the absorption of a mixture of the corresponding mononucleotides (Fig. 1). We propose in the present research to apply this fraction (or percentage) as a quantitative criterion of hypochromicity. We have designated this value as R_E, since it is derived from the ratio R of two extinctions (E), being the extinction of the polynucleotide under examination and the extinction of the corresponding (equivalent) mixture of mononucleotides;

$$R_E(\lambda) = \frac{E_\lambda^{\text{polynucleotide}}}{E_\lambda^{\text{mononucleotide}}}$$

If these extinctions are measured at a wavelength $\lambda = 260\ m\mu$, we represent the ratio as $R_{E(260)}$, and if at $\lambda = 270\ m\mu$, as $R_{E(270)}$.

It may, in exactly the same way, be said that the ratio R_E of a given polynucleotide, at a given wavelength λ is given by the ratio of the optical density D of the corresponding equivalent mixture of mononucleotides at the same wavelength. In other words, the value of R_E is equal to the quotient of the optical density of a preparation and of the optical density of the mixture of mononucleotides, at the same concentration, obtained by complete hydrolysis of the preparation. The ratio R_E, in contradistinction to $\delta(P)$, is not an independent absolute parameter, but represents a quantitative relative measure of hypochromicity, which precisely defines the fraction of the ultraviolet absorption of the given preparation, as compared with the maximum possible absorption of the constituent mononucleotides. With increasing denaturation and degradation of nucleic acids the value of R_E tends towards the unity, and the amount of its change is hence an index of the extent of the denaturation and degradation suffered by the polynucleotide.

For a number of reasons we were obliged to take as the constant standard wavelength for measurement of optical density of our solutions 270 $m\mu$, or the wavelength of maximum absorption by nucleic acids:

$$R_{E(270)} = \frac{D_{270}^{\text{preparation}}}{D_{270}^{\text{mononucleotide}}} = \frac{D_{270}^{\text{before hydrolysis}}}{D_{270}^{\text{after hydrolysis}}}$$

The first reason was that, as the absorption maximum of nucleic acids, the value found for extinction may vary for different preparations, depending on their nucleotide composition. This consideration also applies to measurements of extinction made at 260 $m\mu$. On the other hand, the value of molar extinction of 270 $m\mu$, although somewhat lower than at 260 $m\mu$ or at the absorption maximum, is extraordinarily constant. This constancy may be ascribed to the circumstance that the values of the extinction coefficients of the various nucleosides of nucleic acids are closest to each other at this wavelength [2].

Hence, the sum of the extinctions per mononucleotide at 270 $m\mu$ will vary very little, however much might differ the ratios of the components of nucleic acids. Conversely, molar extinction at 260 $m\mu$ would fall appreciably in the case of a GC-type of polynucleotide (i.e., one in which there is an excess of guanylic and cytidylic acids, as compared with adenylic and uridylic acids), and would rise with transition to an AT-type of DNA, or an AU-type of RNA.

A second important consideration was that the magnitude of extinction at 270 $m\mu$ is relatively independent of the nature and extent of hydrolytic degradation of nucleic acids, in contrast to its magnitude at the extinction maximum or at 260 $m\mu$. For purposes of comparison, absorption curves for alkaline (0.5 N NaOH; 18 hr at 37°) and acid (0.5 $NHClO_4$; 15 min at 100°) hydrolyzates of RNA are shown in Fig. 2A. Theoretically, this form of acid hydrolysis cannot be applied for the purpose of deriving R_E values, since it involves partial destruction of purine nucleotides, while not always effecting hydrolysis of internucleotide links between pyrimidine nucleotides. Nevertheless, the values found for extinction of the hydrolyzates at 270 $m\mu$ are very close to those found for an authentic mixture of mononucleotides; it can, therefore, be used instead of the slower alkaline hydrolysis for the derivation of R_E, but only at a wavelength of 270 $m\mu$. The same considerations apply to the case of DNA, where the values of the extinction coefficients at 270 $m\mu$ of a mixture in the theoretical proportions of desoxyribonucleotides are remarkably close to those found directly in the acid hydrolyzate (0.5 N $HClO_4$; Fig. 2B).

For the above reasons, we have in this research applied the ratio $R_{E(270)}$ in the spectrophotometric evaluation of hypochromic behavior. For purposes of illustration we present (Table 1) the values of this ratio for various DNA and RNA preparations, together with the values of their $\delta(P)$ at 260 and 270 $m\mu$, and with the $R_{E(260)}$ values. All the data were derived from measurements of solutions in phosphate buffer of pH 7.2 and ionic strength $\mu = 0.1$.

TABLE 1

Values of some Spectrophotometric Indexes of Nucleic Acids

Nature of preparation	$\varepsilon(P)_{260}$	$R_{E(260)}$	$\varepsilon(P)_{270}$	$R_{E(270)}$
Native DNA	6500	0,60	5350	0,56
Denatured DNA	7060	0,65	5900	0,61
Native RNA (from TMV)	78 0	0,69	6050	0,65
Yeast RNA (alkaline extraction)	~9700	~0,88	~8000	~0,86
Yeast RNA (commercial)	88 0—	0,80—	7200—	0,77—
	—10200	—0,93	—8400	—0,90

TABLE 2

Increase in R_E and $\delta(P)$ Associated with Denaturation of DNA

Solution under examination	$R_{E(270)}$	$R_{E(260)}$	$\varepsilon(P)_{260}$
Solution of DNA in 0.003 M NaCl	0,56	0,60	6 00
Solution of DNA in 0.003 M NaCl, after heating for 1 hr at 100°	0,74	0,76	8220
Solution of DNA in 0.01 N HCl	0,79–0,82	0,79–0,83	8230–8620
Solution of DNA in 0.1 N NaOH	0,78	0,81	8160

RESULTS OF EXPERIMENTS

Since more is kown about the structure and properties of desoxyribonucleic acids and their denaturation and degradation products than of other polynucleotides, we confined our examination of hypochromic behavior to this material. With this object, we undertook a comparative spectrophotometric study of native and denatured DNA and of the products of its specific degradation.

Comparative Study of Native and Denatured DNA

Denaturation of native DNA is associated with quite considerable increase in $\varepsilon(P)_{260}$, and accordingly in R_E, by 25-45% [2, 7-10]. It has been shown that this effect is due to rupture of hydrogen bonds joining the bases of the two parallel chains of the molecule [2, 9, 10, 25]. We found that the $R_{E(270)}$ of native DNA rose to values of 0.75-0.80 after heating for 1 hr at 100° in 0.003 M NaCl, with addition of acid or alkali. Some of the data are presented in Table 2.

If, however, the solution of DNA, treated in any of the above ways, is made neutral, and its ionic strength is raised to $\mu = 0.1$, considerable, although not complete, reversion to the initial absorption is observed, $R_{E(270)}$ falling to about 0.6, and $\varepsilon(P)_{260}$ to about 7000 (Fig. 1). For example, immediate neutralization of a solution of DNA in 0.01 N HCl, with raising of the buffer strength to $\mu = \sim 0.1$, caused a fall in the value of $R_{E(270)}$ of from 0.79 to 0.59; similar treatment of a solution of DNA in 0.1 N NaOH converted the $R_{E(270)}$ to a value of 0.62. A solution of a specially prepared fully denatured specimen of DNA, of pH 7 and ionic strength 0.1, also had $R_{E(270)} = 0.61$ (Table 1). All this leads to the conclusion that after denaturation, and in neutral solution of high ionic strength, the free un-ionized basic groups again interact, to yield one or another reconstituted form of the polynucleotide chains.

Thus, in solutions of sufficiently high ionic strength, both native and denatured preparations of DNA exhibit considerable hypochromicity, due to the formation of hydrogen bonds between the bases of the polyneucleotide chains. The values of R_E and $\varepsilon(P)$ are always higher for denatured than for native DNA, but the difference is not great: $R_{E(270)} = 0.56$ for native, and 0.61 for denatured DNA, and $\delta(P)_{260} = 6500$ for native, and 7000 for denatured DNA (Table 1). Thus the magnitude of hypochromicity is very similar in both cases.

However, a study of the hypochromic behavior of the two preparations under different conditions clearly revealed differences in the nature of their hypochromicity. Fig. 3 illustrates the dependence between the value of $R_{E(270)}$ and the pH of the solutions of native and denatured DNA. All the measurements were made in buffer solutions of identical ionic strength (0.1) (citrate buffer for pH up to 4.5, acetate buffer for the range pH 5-5.5, and phosphate buffer around the neutral point). It is evident from Fig. 3 that no significant changes take place in the UV absorption of native DNA over the pH range 3-7. Only at pH values below 3, i.e., with ionization of the amino group of the guanine residues (pK_a, = 2.75), and with consequent denaturation of the DNA [9, 25], was there a very abrupt rise in UV absorption; $R_{E(270)}$ rose to a limiting value of 0.82, at which it remained with further fall in pH. Analogous changes in the intensity of UV absorption of native DNA, with change in pH of the solutions have been reported by other authors [9]. The reason for these changes lies in the breaking of all the hydrogen bonds linking together the chains of the native DNA molecule.

Hence the changes in the values of $R_{E(270)}$, from 0.56 (for the initial structure of hydrogen bonds) to 0.82 (when all the hydrogen bonds linking parallel chains are broken) are due to the interactions between pairs of bases present in adjacent chains – adenine with thymine, and guanine with cytosine.

The behavior of denatured DNA when the pH is varied is of a different nature. Intensification of UV absorption here begins at pH 4.0, and becomes maximum ($R_{E(270)}$ = 0.8) at pH 3. The limiting value of $R_{E(270)}$ was very close to that for native DNA (0.8). Taking the pK_a, of the amino groups of cytosine and adenine as being about 4.5 and 3.5, respectively [2, 20], a correlation can be distinguished between ionization of these amino groups and intensification of UV absorption. If we regard solutions of native DNA as representing populations of pairs of chains, firmly linked together by hydrogen bonds, and forming regular double helices, then denatured DNA solutions would represent assemblies of randomly distributed single chains, which under the given conditions (pH 7; μ = 0.1) yield chains and complexes which are associated in a random and disorderly manner. To the random and disorderly manner of bonding is due the much smaller rigidity of the whole structure, and hence the greater lability of all the bond systems. Even at low acidities, ionization of the remaining free amino groups, in the first place of cytosine, causes augmentation of repulsive forces acting between associated chains, leading to rupture of hydrogen bonds linking them, and to the appearance of fresh free amino groups, and so on. To this process is due the gradual nature of the intensification of UV absorption of denatured DNA with lowering of the pH of the medium.

The labile and disorderly nature of interchain associations of denatured DNA, as compared with the rigid, orderly structure of native DNA, can readily be perceived from a comparison of the effect of varying the ionic strength on the value of $R_{E(270)}$ (Fig. 4). Raising the ionic strength over the range 0.001 to 1 had no effect whatsoever on the $R_{E(270)}$ of native DNA. With denatured DNA, on the other hand, $R_{E(270)}$ was found to vary inversely with the ionic strength, e.g., from 0.64 at μ = 0.01 to 0.61 at μ = 0.1, and to 0.59 at μ = 1. This diminution of the R_E values may readily be related to increase in disorderliness of interchain linkages, due to screening of similarly charged groups of the polyelectrolytes in media of higher ionic strength. Because of the weakening of repulsive forces between chains, a larger number of sites on the latter become able to interact. In native DNA, on the other hand, it is obvious that increase in ionic strength cannot cause any rise in the number of reactive basic residues present in the chains.

Confirmation of the lability of the disorderly associations of chains present in solutions of denatured DNA, as compared with the rigid and orderly structure of native DNA, is afforded by the effect of urea on the values of $R_{E(270)}$ (Fig. 5); whereas the absorption of native DNA is only very slightly affected by dissolving the latter in 6 M urea solution (20°), the $R_{E(270)}$ of denatured DNA shows a considerable rise, to 0.70, under these conditions.

A comparison of the hypochromic behavior of native and denatured DNA thus leads to the conclusion that the hypochromicity of polynucleotides is to a certain extent due to the structure of the system of hydrogen bonds (or of other, similar bonds) interlinking basic residues of chains or segments of chains (macromolecular hypochromicity). These bonds may be primary ones, specific to the original, native structure of the molecule, or they may be formed secondarily, in different ways, according to the conditions prevailing at the time of their formation. We may therefore distinguish two forms of hypochromicity – that due to the native macrostructure of the molecule, and that due to secondary formation of association between chains.

However, when absorption rises following rupture of hydrogen and secondary bonds of DNA, the R_E values found are always less than unity. The absorption found for DNA solutions under conditions ensuring rupture of

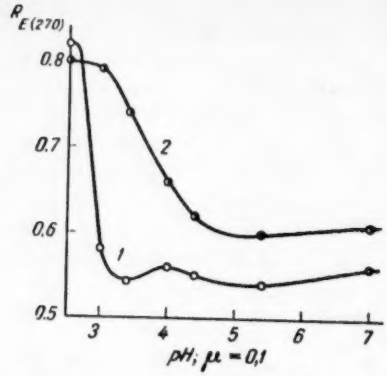

Fig. 3. Spectrophotometric titration of native and denatured DNA: relationship between the $R_{E(270)}$ value and the pH of the solutions at constant ionic strength (μ = 0.1). 1) native DNA; 2) denatured DNA.

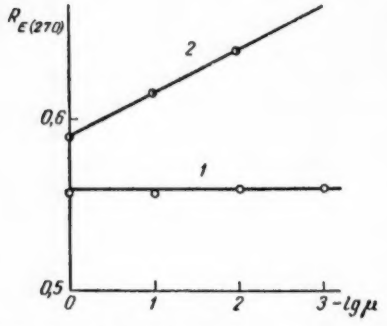

Fig. 4. Effect of ionic strength on absorption of native and denatured DNA: dependence of $R_{E(270)}$ on the ionic strength of phosphate buffer of pH 7. 1) native DNA; 2) denatured DNA .

all such bonds never exceeds a certain limiting value of about 0.8, irrespective of the means employed for breaking the bonds (Table 2, Figs. 3 and 5). It follows that the hypochromicity of polynucleotides cannot be due exclusively to the presence of any form of interchain linkage. We shall term the contribution made to hypochromicity by other factors "residual hypochromicity." Some light is thrown on the nature of these other factors by the results of our examination of the DNA derivatives APuA and APyA.

Comparative Study of APyA, APuA and DNA

Inasmuch as APyA is a DNA chain from which all the pyrimidine bases have been eliminated, and APuA is the same chain, from which all the purine bases have been removed, it follows that in neither of these products can there be any possibility of coupling of adenine with thymine, of of guanine with cytosine. Moerover, each of these products represents a simplified model of the desoxyribopolynucleotide molecule, permitting of the study of the properties of purine or pyrimidine nucleotides present in polynucleotide chains, in separation from each other. Each of the models fully complements the other one. We therefore carried out comparative studies of these two products and of the DNA from which they were prepared, with the object of ascertaining: 1) whether adenine is capable of reacting with guanine, or cytosine with thymine, i.e., whether interpurine or interpyrimidine bonding of chains is possible (analogous to the known purine – pyrimidine bonding); 2) whether "residual hypochromicity" is associated with either the purine or the pyrimidine residues of the chains.

Evaluation of its $R_{E(270)}$ in phosphate buffer of pH 7 and μ = 0.1 gave quite high values for APuA, amounting to 0.90. Contrary, however, to certain published reports [23], the absorption found by us for APuA was smaller than for a corresponding mixture of desoxycytidylic and thymidylic acids. Thus, APuA exhibits a small, but significant, hypochromicity. Its value, as expressed by $R_{E(270)}$ = 0.90, is quite constant, and is dependent of the ionic strength (from 0.01 through 1), of the pH (from 1 through 13), and of the presence of urea (6 M solution). The conclusion may hence be drawn that the hypochromicity of this product consists entirely of "residual hypochromicity", and is not dependent on any form of labile interchain reactions between pyrimidine residues. Variations in the degree of ionization of the cytidine or thymine residues had no effect whatsoever on the R_E value of APuA. This evidence totally excludes any possibility of formation of hydrogen bonds between the pyrimidine bases of different chains, and leads to the conclusion that residual hypochromicity must be due to some other factors.

The hypochromic behavior of APyA (Fig. 6) differed considerably from that of APuA. It had much lower $R_{E(270)}$ value (0.69), in buffer of pH 7 and μ = 0.1. This value was even lower in NaCl solution of the same ionic strength, being only 0.64. Thus, APyA exhibits considerable hypochromicity, although possesses no pyrimidine residues, and hence cannot form hydrogen bonds of the adenine – thymine and guanine – cytosine types. The $R_{E(270)}$ value was raised only slightly, to 0.73, in 6 M urea solution. Ionization of the basic residues had, however, a much greater effect on the UV absorption of APyA. Thus, $R_{E(270)}$ rose more or less smoothly to a limiting value of 0.78, as the pH fell from 5 to 2.5 (μ = 0.1). A considerable rise in absorption was also found when APyA was dissolved in 0.1 N NaOH, $R_{E(270)}$ rising to the same limiting value of 0.78. It might hence be thought that part of the hypochromicity of APyA could be due to interactions between chains, acting through the purine residues. We cannot therefore exclude the possibility of formation of hydrogen bonds between polynuc-

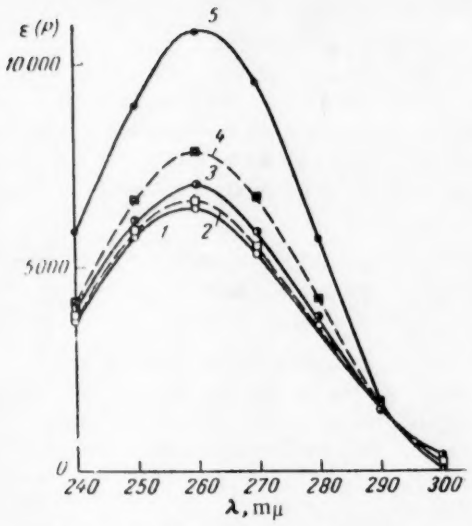

Fig. 5. Effect of urea on the absorption curves for native and denatured DNA, at pH 7 and 20°; 1) native DNA in buffer solution of μ = 0.1; 2) native DNA in 6 M urea solution; 3) denatured DNA in buffer solution of μ = 0.1; 4) denatured DNA in 6 M urea solution; 5) a mixture of free desoxyribonucleotides.

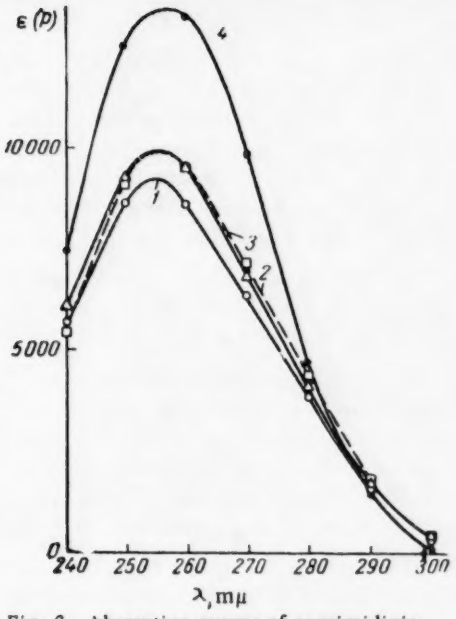

Fig. 6. Absorption curves of apyrimidinic acid (APyA) under various conditions, and of a corresponding mixture of purine desoxyribonucleotides, at pH 7. 1) APyA in NaCl solution, μ = 0.1; 2) APyA in phosphate buffer, μ = 0.1; 3) APyA in 6 M urea solution, 20°; 4) theoretically equivalent mixture of desoxyguanylic and desoxyadenylic acids, in the proportions G : A = 1 : 1.

leotide chains, not involving pyrimidine bases, but due to purine bases only; such linkages could be of the "purine – purine" type, or of the "purine – phosphate" type (in the latter case, for example, between guanine amino groups and unionized oxygen atoms of phosphate diesters).

This mechanism could, however, account for only part of the hypochromicity of APyA which is abolished by ionization; the persistent "residual hypochromicity" of this compound is fairly considerable. It is much greater than for APuA, and it even exceeds that of the original DNA. A comparison of the magnitude of the "residual hypochromicity" of the original DNA ($R_{E(270)}$ = 0.8) with that of APyA ($R_{E(270)}$ = 0.78) and of APyA ($R_{E(270)}$ = 0.9) suggests that the greater part of the "residual hypochromicity" of DNA may be due to its purine nucleotides.

Mention should, in this connection, be made of three groups of data obtained from other fields of research in polynucleotides.

The first of these groups relates to the study of the optical properties of the products of reacton of RNA with pancreatic ribonuclease. The resistant core remaining after dialysis of the enzymatic hydrolyzate of RNA, and consisting basically of polypurine oligonucleotide segments, was found to exhibit high hypochromicity [1, 14]. It appears from Reddi's data [14] that its hypochromicity amounted to about 60% of that of the RNA taken for the experiment. The author hence concluded that the hypochromicity of RNA depends less on the length of the polynucleotide chain than on the presence in the chain of segments consisting of sequences of two or more purine nucleotides (poly purine nucleotide segments).

The second group relates to researches on synthetic polynucleotides, prepared by the action of polynucleotide phosphorylase. Polyadenylic acid (poly-A, or A-polymer) was found to exhibit quite considerable hypochromicity, in contrast to polyuridylic acid (poly-U, or U-polymer), the hypochromicity of which was negligible [3, 4]. Although considerable hypochromicity was shown by polycytidylic acid (poly-C or C-polymer), its

value was lower than for poly-A [4].

Similar studies have not yet, unfortunately, been carried out for polyguanylic acid. It may, however, be concluded from the data at our disposal that the poly purine nucleotide chain as such is distinguished by its high hypochromicity, and that its hypochromicity is higher than that found for the pyrimidine nucleotide chain. The third group of data relates to the optical properties of dinucleotides. These have been found to exhibit a certain degree of hypochromicity, which obviously cannot be related to the presence of any form of hydrogen bonding, or to any reactions between molecules. The most pronounced hypochromicity was found for the dinucleotides containing purine bases [5, 6, 15].

It is evident from our results, and from the published data, that "residual hypochromicity" is not related to formation of intermolecular hydrogen bonds, but that it results from the polymerization of nucleotides. The greater part of residual hypochromicity can be attributed to the polymerization of purine mononucleotides.

Inasmuch as the absorption of nucleotides could scarcely be thought to be affected by the formation of diesteratic internucleotide bonds, as such, we assume that in the given case the observed fall in ultraviolet absorption may be due to some form of interaction between the parallel planes of adjacent purine or pyrimidine ring systems. The degree of interaction between the planes of purine rings greatly exceeds that between those pyrimidine rings, possibly because of the larger dimensions of the former, which may provide more points of contact in directions perpendicular to the planes of the rings, i.e., along the chains.

We make the assumption, based on the above considerations, that the "residual hypochromicity" of DNA, the hypochromicity of APuA and partly of APyA, of dinucleotides, and a large proportion of that of artificial one-component polynucleotides (poly-A, poly-C, etc.) and of the hypochromicity of the core remaining after dialysis of the enzymatic hydrolyzate of RNA are all due to such interactions between the planes of adjacent bases along the polynucleotide or oligonucleotide chains.

The hypochromicity of APyA and APuA, which cannot be related to the existence of hydrogen bonds between chains, should be attributed to the presence in the desoxyribose phosphate chains of considerable numbers of di-, tri- and poly purine or pyrimidine nucleotide segments (blocks). It has been shown that such blocks of nucleotides are a characteristic feature of the nucleotide sequences of DNA [26, 27]. It may, from a comparison of the data for APyA and APuA, be concluded that the greater part of the "residual hypochromicity" of DNA is due to the presence of poly purine nucleotide segments in the DNA chain.

SUMMARY

For the quantitative expression of the "hyperchromic effect" of polynucleotides we propose the coefficient $R_{E(270)}$, being the ratio of the optical density of a given polymer at 270 mμ to that of the corresponding mixture of free mononucleotides, at the same wavelength:

$$R_{E(270)} = D_{270}^{\text{polynucleotide}} \Big/ D_{270}^{\text{mononucleotides}}$$

The application of this coefficient to the study of DNA showed that the smaller UV absorption found for polynucleotides, as compared with that of a mixture of the constituent mononucleotides (which constitutes the hypochromicity of polynucleotides), is due to a number of factors associated with the structure of the polynucleotides, and is made up of the hypochromicity contribution attributable to the presence of hydrogen bonding between chains or segments of chains, and of the "residual hypochromicity", which is an effect of the polymerization of nucleotides. A study of the apurinic and apyrimidinic acids derived from DNA showed that most of the residual hypochromicity" of DNA is attributable to the polypurine nucleotide segments present in the chain.

Our results suggest that at least three kinds of structural factors contribute to the magnitude of the UV absorption of polynucleotides; these are: the presence of hydrogen bonding between purine and pyrimidine bases in the two chains of the native DNA molecule, the formation of randomly located hydrogen bonds between purine and pyrimidine bases of chains or segments of chains (applying particularly to denatured DNA), and interaction between the planes of adjacent purine or pyrimidine rings, being a consequence of the polymerization of the mononucleotides.

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THE ROLE OF ACTIN IN MUSCLE TISSUE AND SOME QUESTIONS OF THE THEORY OF MUSCLE CONTRACTION

M. M. Zaalishvili and G. V. Mikadze

Institute of Physiology, Georgian SSR Academy of Sciences, Tbilisi

As the investigations of Engel'gardt and Lyubimova [1,2] have shown, the reaction of the muscle protein myosin with ATP lies at the base of the contraction process of muscle. The observation of the adenosine triphosphatase nature of myosin and the effect of ATP on the mechanical properties of the myosin fibers form the basis for the ideas which Engel'gardt has worked out concerning the "actor-catalyst" [3]. From this point of view, myosin combines in itself the function of an enzyme and a contracting substance.

In 1942 the Hungarian biochemists discovered a new protein in muscle tissue, actin; this led to the idea that the contracting substance was not myosin, but a complex of it with actin, actomyosin [4]. Most of the facts accumulated in later years in model experiments allow us to assume that ATP causes contraction of actomyosin. Hence the hypothesis has arisen that actomyosin is a contractile substance and therefore contraction of actomyosin fibers can be considered the analog of muscle contraction.

In experiments presented earlier, actomyosin fibers were obtained by the method of Weber [5] as perfected by Gerendas and Dubuisson [4]. In such fibers the protein particles have a definite orientation. However, it has been shown that the Weber fibers do not have the mechanical strength which is present in muscle fibers and cannot carry on work [6].

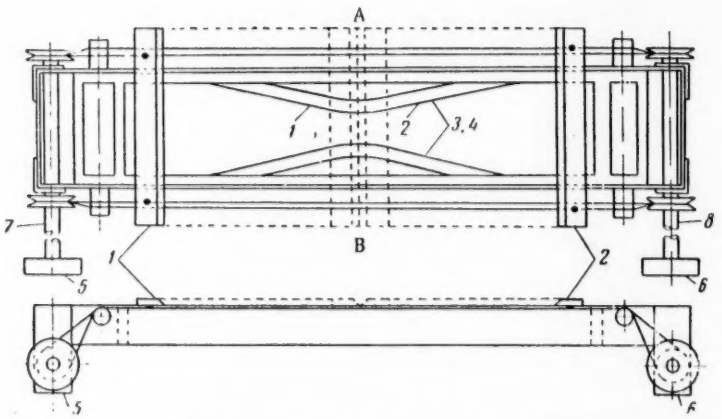

Fig. 1. Langmuir bath for obtaining the film fiber: A) View from above; B) view from the side; 1 and 2) barriers; 1 and 2 in the center of the figure) barriers when the film is compressed; 3 and 4) narrowing rims; 5 and 6) cranks for moving the barriers; 7 and 8) pulleys.

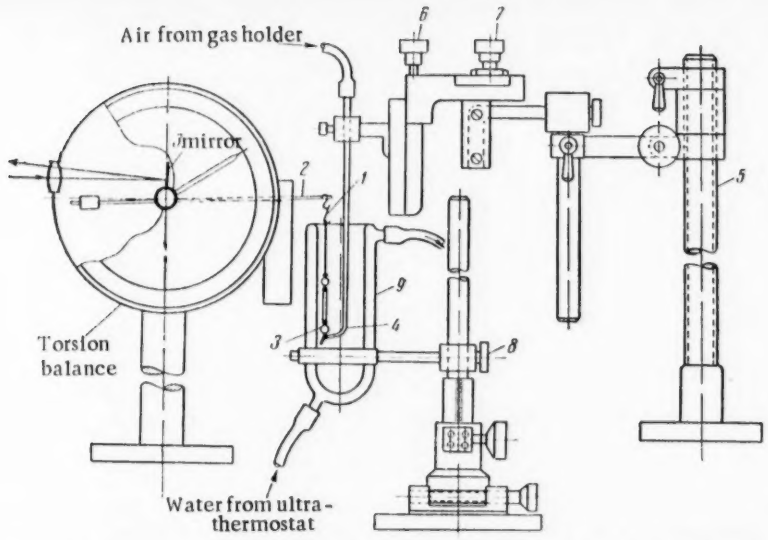

Fig. 2. Construction of the tensimeter: 1) metal hook; 2) lever of torsion balance; 3) heart forceps; 4) capillary tube; 5) support; 6, 7) microscrews; 8) universal clamp; 9) double-walled beaker

In the work of Szent-Györgyi [7] it was shown that actomyosin fibers under the influence of ATP contract to 30% of the starting length; Mg^{++} increases the contraction and Ca^{++} weakens it. The rate of contraction is limited by the rate of diffusion of ATP in the fibers. He showed that under determined conditions, the contraction was reversible.

According to the data of Ivanov [8], when ATP is added the actomyosin fibers thicken, become more resistant to rupture, and their elasticity increases. When they are loaded, they acquire the ability to stretch and without this, to contract. Fibers of pure myosin, according to Erdös [9], do not contract under the influence of ATP.

The contraction of actomyosin fibers which occurs on the addition of ATP [10-12] qualitatively and in some cases quantitatively resembles the contraction of muscle. However, compared to muscle, the fibers are characterized by a low protein content so that the volume of work and the shortening of the fibers is much less than in muscle.

The fibers obtained by drying ordinary fibers are a more satisfactory model; their protein concentration reaches 6-10% [13]. They must be dried under carefully controlled conditions so as to prevent denaturation of the actomyosin.

In recent times a process has been suggested for obtaining protein fibers by compressing a surface film of protein at high pressures [14]. Hayashi [15,16] first studied actomyosin monolayer fibers. He showed that in KCl solutions with an ionic strength of 0.05, 0.03% ATP caused contraction of actomyosin fibers. The contraction of monolayer actomyosin fibers does not have the character of syneresis.

Ashmarin [17] showed that myosin gains the ability to contract under the influence of ATP not only in the complex with actin, but after combination with some dyes. He succeeded in obtaining a sample of myosin to which a dye was added instead of actin. The author concluded from this that actin plays a subordinate role in the contraction process.

According to the data of Ryzhlov and Loidina [18], myosin forms a complex with tobacco mosaic virus. The fibers from this complex contract under the influence of ATP. Vorob'ev [19] found that Weber fibers obtained from the myosin-DNA complex also contracted under the influence of ATP.

In the work of Kafiani [20], who studied the contractile properties of films of myosin fibers, these contracted under the influence of ATP. In the opinion of this author, these facts confirm the viewpoint of the individuality of myosin as a contractile protein. He concluded from the experimental material that actin is not essen-

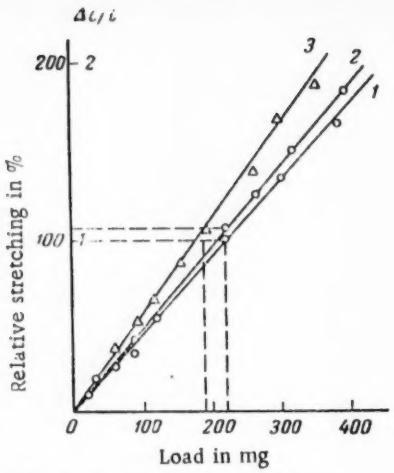

Fig. 3. Effect of ATP on relative stretching of myosin fibers Temperature 20°; pH 7; 1 and 2) myosin fibers in the absence of ATP; 3) myosin fibers in the presence of 0.3% ATP. Composition of the salt solution 0.05 M KCl, 10^{-4} M $MgCl_2$, and 0.02 M potassium-veronal buffer.

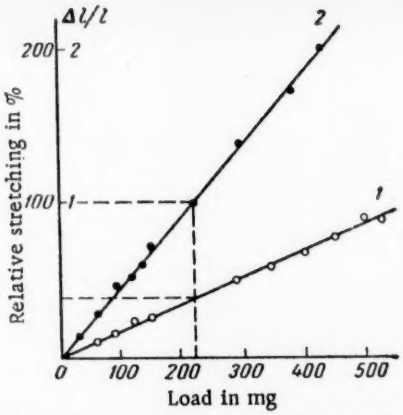

Fig. 4. Effect of ATP on relative stretching of actomyosin fibers. Temperature 20°; pH 7; 1) actomyosin fibers in the absence of ATP; 2) actomyosin fibers in the presence of 0.3% ATP; composition of solution, see Fig. 3.

tial for the contraction of the monolayer fibers. However, these results were disproved in the similar studies of Hayashi and co-workers [21] which showed that fibers of monolayers did not contract with ATP. We came to the same conclusion in 1956 when we showed by special experiments that under the influence of ATP it was not the fibers of pure myosin which shortened, but only of actomyosin [22]. We concluded from this that in muscular contraction both actin and myosin take an active part; actin is not a passive component of muscular contraction.

In the present study we give results which we have obtained by investigating the contractile and mechanical properties of monolayer myosin and actomyosin fibers.

METHODS

Myosin was obtained by the method of Szent-Györgyi [4]. Actomyosin was obtained by the following method: ground muscle was extracted for 24 hours in the cold with three volumes of Guba and Straub solution. The mixture was centrifuged to remove the large particles and the viscous centrifugate was passed through two layers of gauze. The protein extract was again centrifuged to remove the fine particles. The clear extract was treated with 10 volumes of ice water with stirring, and allowed to stand overnight in the refrigerator to permit the precipitate to settle. The upper layer of liquid was removed by decantation. The precipitate which collected in the centrifuge was dissolved in an equal volume of 1.2 M KCl. The resulting protein solution was treated with 1.2 M KCl and glycerol in the proportions 1:1:1. The glycerol solution of actomyosin was kept at -6° without any loss of ATP-ase activity for a week. Actin was obtained by the method of Szent-Györgyi and Guba [4], and ATP was obtained as the dibarium salt by the method of Kerry [23]. Before use of the ATP its barium salt was converted to the potassium salt.

Obtaining fiber films. To obtain the fiber films we used a Langmuir bath with dimensions 100x15x1 cm. The bath gradually narrowed toward the center, and its width at the center was 3 cm (Fig. 1). Movable barriers were placed at the two opposite ends. The bath and the movable barriers were covered with paraffin. The solution consisted of 0.5 M KCl in 0.02 M veronal-potassium veronal buffer which contained 10^{-4} M MgCl2. The protein solution was introduced onto the surface as a drop with a micropipet. After slowly drawing the barriers together for 5 min, the film was compressed into a fiber which was removed from the bath after two minutes with a dissecting needle. The fiber was held at each end by small steel heart forceps which weighed 3 mg each. Such a bath construction allowed us to obtain very strong fibers. The average length of the fibers was 18-22 mm and the width 0.7 mm.

TABLE 1

Effect of ATP on the Strength of Myosin and Actomyosin Fibers

Object studied	Average value of maximum load at which breaking of the fibers occurs, in mg			
	Protein in glycerol		Protein in isoamyl alcohol, 1 : 1000	
	without ATP	with ATP	without ATP	with ATP
Myosin fibers	1090	675	3600	2030
	550	5 0	590	630
Actomyosin fibers	6.0	420	700	600
	600	320	710	620
	680	400	—	—
Average	620	422,5	700	617

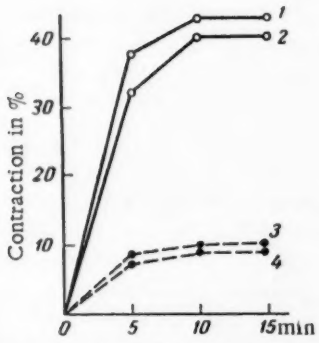

Fig. 6. Effect of ATP on contraction of actomyosin fibers. Temperature 20°; pH 7; 1 and 2) actomyosin fibers in the presence of 0.3% ATP; 3 and 4) actomyosin fibers in the absence of ATP.

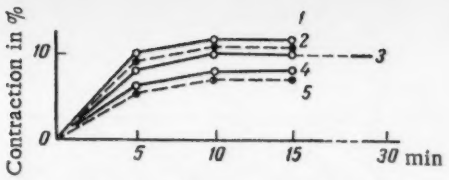

Fig. 5. Effect of ATP on contraction of myosin fibers. Temperature 20°; pH 7; 1 and 4) myosin fibers in the presence of 0.3% ATP; 2 and 5) myosin fibers in the absence of ATP; 3) contraction of myosin fibers which were first kept in a salt solution, in the presence of 0.3% ATP.

Construction of the tensimeter. The tensimeter was constructed on the principle of a torsion balance(Fig. 2). It differed from the apparatus of Engel'gardt and Lyubimova [2] in that the recording of the change in length of the fiber was carried out by the shift in the ray of light reflected from a mirror fastened to the axis of rotation of the beam of the torsion balance (and not to the lever arm),and also by direct observation with a cathetometer. Since the distance between the mirror and the scale was 1.5 m, a slight change in length of the fiber caused a great variation on the scale. This process permits more accurate estimation of the shortening of the fiber.

The protein fiber was fastened at one end by a heart forceps and the other end was attached to a fine silk thread treated with vaseline, 4 cm long. This was attached to a metal hook (Figs. 1 and 2) by which the protein fiber was suspended on the lever arm of the torsion balance, 2. The bottom end of the film fiber was held by the heart forceps 3 to the curved end of the capillary tube 4. The capillary tube was held by a guide ST-12 which was mounted vertically on a separate support. By turning the microscrews 6,7,the capillary tube could be moved horizontally and vertically so that the film fiber was adjusted without stretching (in this case the torsion balance was fastened down).

The two walled beaker 9, volume 25 cm^3, was held by the universal clamp 8; it had two openings for the circulation of water between the walls from the Hepler ultrathermostat. This construction of the apparatus allowed us to carry out experiments at constant temperature, to change it quickly, and to raise the beaker quickly so that the fiber, held between the arm of the balance and the curved end of the capillary tube, could be dipped into a buffered solution of KCl. In order to stir the solution after adding ATP, we passed air from time to time from the gas holder into the solution through the capillary 4.

Conduct of the experiments. At first we measured the length of the upright fiber with the cathetometer. Then we slightly raised the lower end of the fiber by raising the capillary, which permitted us to measure the length without a load. After addition of ATP over a definite period of time, the length of the fiber was measured by the method described. As a control we used fibers dipped in the same KCl solution, but instead of potassium adenosinetriphosphate (ATP-K), we added the same amount of potassium as was found in the ATP-K (in 0.3% ATP-K the concentration of potassium ions was 0.02 M, and so in the control experiments we added 0.02 M KCl instead of 0.3% ATP-K). We subtracted the value for the contraction of the fibers in the control experiments in order to obtain the true value for the contraction caused by ATP.

The apparatus also permits measurement of the amount of contraction without a load in another way: at the lower end of the fiber are the forceps held by paraffin to the curved end of the capillary tube and at the

TABLE 2

Stretching and Residual Deformation of Actomyosin Fibers Depending on Load

Load		Load l_2, mm		$\frac{l-l_0}{l_0} \times 100$	Residual deformation of fibers	
at cross section of fibers (4×10^{-3} cm²) in mg	in g/cm²	load, l_1, mm	load, l_1, mm		l_2-l_1, mm	relative $\frac{l_2-l_1}{l_1} \times 100$
0.0	0.00	16.0	—	—	—	—
30.0	7.50	17.0	16.0	6.25	0.0	0.00
60.0	15.00	18.0	16.0	12.50	0.0	0.00
90.0	22.50	19.0	16.0	18.75	0.0	0.00
120.0	30.00	19.5	16.0	21.87	0.0	0.00
190.0	47.50	21.4	16.0	34.00	0.0	0.00
2.5.0	56.25	22.4	16.0	40.0	0.0	0.00
290.0	72.50	24.6	16.0	54.0	0.0	0.00
340.0	85.00	25.9	17.0	62.0	1.0	6.25
400.0	100.00	27.2	17.5	70.0	1.5	9.37
450.0	112.50	28.5	18.0	78.0	2.0	12.50
500.0	125.00	30.1	19.0	88.0	3.0	18.75

upper end the fiber is attached by the hook to the arm of the balance. Then the balance is adjusted until the fiber is straight, and the length of the latter is measured by the cathetometer. When the cathetometer is set at the level of the upper end of the fiber, the latter is shortened from below. The length of the shortened fiber is measured by the cathetometer. For an exact determination of the contraction of the fiber we can also follow the deflection of the light beam reflected from the mirror fastened on the axis of rotation of the arm of the balance.

Measurement of the contraction of the fiber with a load could be carried out directly by the cathetometer or by deviation of the light beam. For measurement of the contraction of the fiber by the first method, the balance was fixed, and loads of different weights were placed on the forceps which were fastened to the lower end of the fiber. When the length of the fiber changed, the curved part of the capillary followed the movement of the forceps. The length of the fiber with different loads was measured by the cathetometer. For measurement of the length of the fiber with high loads, the lower end of the fiber was temporarily placed at the bend of the capillary. The initial length of the fiber was measured with the cathetometer by the described method; the fiber was raised from the solution by lowering the beaker, its lower end was freed, and loads of different weight were placed on the forceps. The length of the fiber was measured with each load. The fiber was again dipped into the solution and kept in it until the original length was regained. Measurements of stretching were carried out until the fiber broke.

In the second process, the forceps were fastened by paraffin to the curved end of the capillary, and the upper end of the fiber was attached by the hook to the arm of the balance. The fiber was straightened by turning the handle of the balance. The initial length of the fiber was measured; then, by turning the handle, the fiber was given various loads whose size was indicated on the scale of the torsion balance. Either the cathetometer or the light beam could be used to measure the change in length of the fiber at the time. Our apparatus permitted measurement of the change in length of the fiber caused by loading, and also the load which was required to cause a given stretching. Thus, the construction of our tensimeter permitted study of both isometric and isotonic contraction.

Knowing the initial length of the fiber, and measuring its changes, we could determine the relative contraction (or stretching) of the fiber, expressed in percent of the initial length of the fiber.

EXPERIMENTAL PART AND DISCUSSION OF RESULTS

We studied by our method the relation to the load of linear deformation and resistance to breaking in myosin and actomyosin fibers in the presence of 0.05 M KCl, 10^{-4} M $MgCl_2$, and solutions of the same ionic composition with a content of 0.3% ATP. The experiments were carried out at 20° and pH 7 (pH was determined by the LP-5 potentimeter). A glycerol solution of the protein was placed on the surface of the solution, and in some cases, in order to make the spreading of the protein easier on the surface, we also used solutions of the protein which contained isoamyl alcohol (1:1000).

In Figs. 3 and 4 we give curves of the relation of relative stretching of myosin and actomyosin fibers with loads at 20° and pH 7 in the presence and absence of ATP. In Table 1 are given the values of resistance to breaking of myosin and actomyosin fibers in the presence and absence of ATP.

The modulus of elasticity for the protein film fibers is calculated by the Hooke formula

$$E = F \cdot \frac{l_0}{\Delta l} \tag{1}$$

where E is the modulus of elasticity, F the stretching force in kg/cm^2, $l_0 / \Delta l$ the reciprocal of the relative stretching of the fiber. When the modulus of elasticity is calculated, the question arises whether the length of the fiber l returns fully to l_0 when the action of the stretching force ceases, that is, whether the process occurs without residual deformation. If this is so, then we are dealing with elastic deformation. We must not forget that in stretching, the length of the fiber increases from l_0 to l so that the cross section is decreased from S_0 to S. This decrease must be taken into account in calculating F in cm^2. If the volume of the fiber remains constant, than $l_0 \cdot S_0 = l$ and then $S = S_0 l_0/l$. However, change in cross section of the fiber is difficult to calculate, so that in calculating the modulus of elasticity the force was related to the original cross section.

As Table 2 shows, with loads of less than 85 g/cm^2, actinomyosin fibers stretch without residual deformation; the stretching reaches 50-60%. Up to this loading, the deformation strictly follows Hooke's Law, which permits calculation of the modulus of elasticity. On the basis of this assumption we calculated E at low loading values.

As Figs. 3 and 4 show, actomyosin fibers are characterized by higher elasticity (Fig. 4, curve 1) than are myosin fibers (Fig. 3, curve 1). This means that actomyosin fibers are more resistant to the stretching force than the myosin. It follows from the results of these experiments that actin strengthens the bonds between the myosin molecules in actomyosin. The modulus of elasticity of actomyosin fibers, calculated by formula (1), is 14.02×10^{-2} kg/cm^2. This value is 2.5 times greater than the value for the modulus of elasticity of myosin fibers (5.6×10^{-2} kg/cm^2).

It is interesting to compare the actomyosin fibers with muscle fibers in respect to their ability to contract and stretch, and also their residual deformation. Living muscles in the equilibrium state can be stretched considerably without residual deformation. They may reach 145% of the resting length. The relative stretching in the present case is 45%.

In Table 2 we give values for the development and disappearance of deformation of actomyosin fibers when loaded and unloaded. As Table 2 shows, actomyosin monolayer fibers with a diameter of 0.7 mm and a cross section of 4×10^{-3} cm^2 will maintain an average load of 0.62 g, that is, the strength of the fibers is 160 g/cm^2. Muscle contains about 10% actomyosin, and the actomyosin monolayer fibers, about 4% protein. We can assume that if the fibers should contain 10% protein, then the protein fibers wtih a cross section of $1 cm^2$ would support $160 \times 10/4 = 400$ g/cm^2 tension. This value approaches the value of the resistance of muscle to breaking. Thus the sartorius muscle, with a cross section of 0.6 cm^2 supports a load of 350-400 g. Thus, actomyosin fibers resemble muscle in this respect. It is true that these calculations have an approximate character, but on the basis of the experiments which we have carried out, we can conclude that the mechanical properties of muscle (elasticity, resistance to breaking, etc.) are determined chiefly by the properties of the structural proteins, myosin and actin.

ATP decreases the elasticity of actomyosin fibers (Fig. 4, curves 1 and 2) from 14.02×10^{-2} to $5.62 \times$ $\times 10^2$, that is, 2.5 times, while the elasticity of myosin fibers under the influence of ATP is hardly changed (Fig. 3, curves 1, 2, and 3). The slight change in modulus of elasticity of the fibers under the influence of ATP can be explained by the fact that the myosin preparation always contains small amounts of actin. The decrease in strength and elasticity of actomyosin fibers under the influence of ATP is produced because ATP decreases the intermolecular links in actomyosin or causes dissociation of the actomyosin complex into actin and myosin.

Thus, the role of actin is to strengthen the bonds between the molecules of myosin in the formation of a structure, and ATP under these conditions, on the contrary, decreases these bonds and causes a physicochemical change in the structure of the contracting substance. As we will see further, the role of actin is not limited to structure formation.

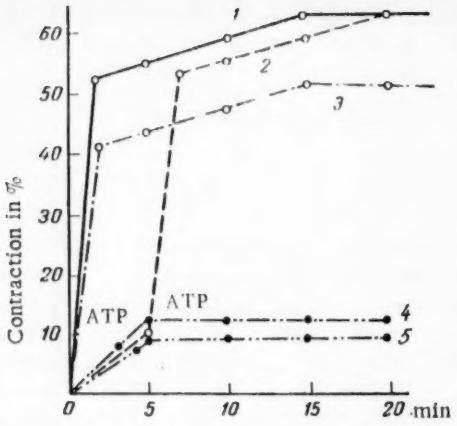

Fig. 7. Effect of ATP and traces of Ba on shortening of actomyosin fibers. Temperature 20°; pH 7.6; 1) actomyosin fibers in the presence of 0.3% ATP; 2) actomyosin fibers in the presence of 0.3% ATP added after 5 min keeping of the fibers in a potassium-veronal buffer; 3) true contraction of actomyosin fibers calculated from the control (curve 5); 4) contraction of actomyosin fibers caused by traces of Ba; 5) contraction of actomyosin fibers in the absence of ATP.

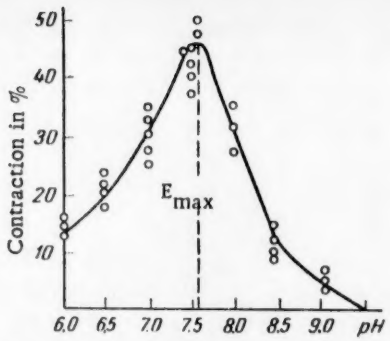

Fig. 8. Relation of shortening of actomyosin fibers to pH of the medium. Temperature 20° in the presence of 0.3% ATP.

Before beginning the study of the contraction of actomyosin fibers, we carried out a careful investigation of the contracting power of myosin fibers prepared by the method described above. The contraction of the myosin fibers took place in the presence of 0.05 M KCl, 0.0001 M $MgCl_2$, potassium-veronal buffer (0.02 M) and 0.3% ATP at different pH values. Since in the experiments we did not use adenosine triphosphoric acid, but its potassium salt, then to exclude the effect of potassium ions on the amount of contraction, we ran the control experiments described above. The results of the experiments run at 20° and pH are given in Fig. 5. Fig. 5 shows that in the presence or in the absence of ATP, shortening of the myosin fibers is almost the same. This led to setting up experiments to study the effect of ATP on the contracting power of myosin fibers which had previously been kept in a solution which contained 0.07 M KCl, 0.0001 M $MgCl_2$, and a veronal buffer (0.02 M), 0.3% ATP. In this series of experiments the myosin fiber was first placed in this solution, and the dynamics of its contraction were recorded for 15 min (the lessening in length); then it was quickly transferred to a solution which contained 0.05 M KCl, 0.0001 M $MgCl_2$, a veronal buffer (0.02 M), 0.3% ATP, and again the length of the fiber was measured during 15 min (Fig. 5, curve 3). As curve 3 shows, in the buffer solution the fiber shortens by 8-12%; when ATP is added, no further shortening is observed. This experiment confirms the observation of Hayashi and co-workers [21] that myosin fibers cannot shorten under the influence of ATP. In the experiments with ATP at pH > 7 we observed not contraction, but on the contrary, stretching of the fibers if they were under a load.

While myosin fibers do not contract under the influence of ATP, actomyosin fibers with ATP at pH 7 contract by 30-36% (Fig. 6). In a buffer solution without ATP, because of changes in temperature, concentration, etc., there was contraction, with values varying from 6 to 10%. Therefore, in this case, as in the case of the myosin fibers in the presence of ATP (Fig. 6, curves 1,2) we subtract the value of contraction of the fibers in a medium which does not contain ATP (control experiments, Fig. 6, curves 3 and 4). As a result we obtain the true curve of contraction, which agrees with the curve of contraction of the fibers in the presence of ATP added after keeping the fibers for 5 min. in a KCl-veronal buffer (Fig. 7, curves 2 and 3).

To test the assumption that the contraction of actomyosin fibers is connected with ATP-ase activity of the actomyosin, we measured the contraction of actomyosin fibers at different pH values and at different ATP concentrations. We showed that the degree of contraction of the fibers depends to a considerable extent on pH. In Fig. 8, we give curves of relation of contraction of the fibers to pH. As these curves show, the optimum pH is about 7.6, that is, in the zone of the optimum adenosinetriphosphatase activity of actomyosin. Fig. 9 shows that the contraction of actomyosin fibers increases with increased ATP concentration. The strongest contraction occurs in the presence of 5×10^{-3} M ATP.

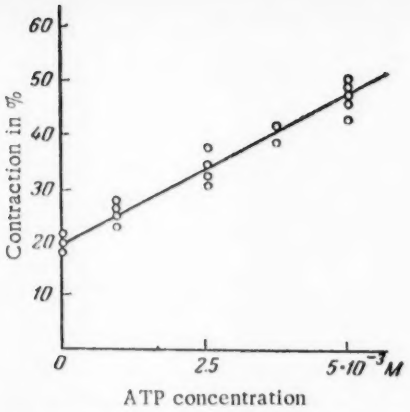

Fig. 9. Relation of shortening of actomyosin fibers to ATP concentration. Temperature 20°, pH 7.6

According to Heilbrun [24] contraction of isolated muscle fibers can be caused by barium ions, since in the process of preparing ATP, barium acetate is used, and complete removal of the barium when ATP-Ba is converted to the potassium salt is very difficult. According to the data of this author, some commercial preparations of ATP, free from barium, do not cause contraction of isolated muscle fibers. We ran special experiments to test the effect of barium on monolayer fibers of actomyosin. For this purpose we prepared a 5×10^{-3} M solution of $BaCl_2$ (this amount of Ba was contained in 0.3% ATP), and for precipitation of the barium we added the necessary amount of K_2SO_4 to the solution; after separation of the barium sulfate precipitate, the centrifugate was added to the monolayer fibers instead of ATP. The results of this experiment are shown in Fig. 7, curve 4. As the figure shows, traces of barium ion can cause contraction of the monolayer fiber by 12-14%, but this value is only 4% greater than the contraction of the fibers in the control experiments (Fig. 7, curve 5). It follows from this experiment that the traces of barium which can remain in the ATP solution after precipitation of the barium give very slight contraction (4%) as compared to the contraction of the monolayer fiber under optimum conditions (40-50%). Direct addition of 10^{-4} - 10^{-3} M $BaCl_2$ produces no noticeable contraction of the monolayer fiber. Thus, contraction of the monolayer fiber does not depend on traces of barium, but on ATP.

According to Mommaerts [6] and Szent-Györgyi [7], the mechanical properties of muscle are related to the presence in it of ATP. In the absence of ATP, muscle loses its elasticity and becomes rigid. Bozler [25] and Szent-Györgyi [26] also observed that elasticity and rigidity of washed muscle fibers decreased under the action of ATP. Therefore, a true analog of muscle would be a model protein system which would undergo the same changes under the influence of ATP. As we saw, the monolayer actomyosin fibers behaved like muscle fibers in this respect.

Analysis of the literature and our own experimental results show that the function of actin chiefly consists in assuring strong intermolecular bonds in actomyosin with the formation of a stable, unbreakable special structure which can change its mechanical properties under the influence of ATP.

Our experiments show that monolayer myosin fibers do not contract with ATP, while actomyosin contracts 50-55% under the influence of ATP. The mechanical effect depends on the ionic strength of the KCl-veronal buffer used in the experiment, on the pH, on the ATP concentration, etc., that is, on those factors which are required for maximum rate of the reaction between ATP and the enzyme, adenosinetriphosphatase. According to our preliminary data, the development of contraction in actomyosin fibers occurs parallel with the accumulation of inorganic phosphate, which is split by the enzyme from ATP. In the first 2 min, contraction of the fiber reaches 40%, and further shortening develops slowly. The splitting of inorganic phosphate from ATP also occurs most intensively at the beginning of the enzyme reaction (in 2 min). All this indicates that contraction of a monolayer actomyosin fiber is caused by the splitting of ATP and by the liberation of energy from this macroenergetic compound.

As we remarked above, the idea that actin plays a secondary role in muscle contraction has been developed by Ashmarin [17] and also by Kafiani [20]. Our results and the existing literature data indicate that lack of recognition of the role of actin in the contraction process is wrong. We assume that the facts established by Ashmarin, and also by Ryzhkov and Loidina [18] and by Vorob'ev [19], do not lessen at all the primary role of actin in muscle contraction. Such investigations only permit study of the mechanism of specific reactions of myosin with actin in simplified model systems. As to the results of Kafiani [20], we should remark that, as Hayashi and co-workers showed [21], contraction of myosin fibers does not occur under the influence of ATP. Actually, if we subtract from the value of the contraction of myosin fibers in the presence of ATP (Fig. 5, curves 1,4) the amount of contraction which occurs in the control experiment (curves 2,5), then the contraction caused by ATP approaches zero.

From all the above, we can conclude that actin in muscle has two functions: 1) to assure strong intermolecular bonds in actomyosin with formation of a mechanically stable structure; 2) it is the active component in contraction. Only the actomyosin system with an unbroken spatial structure can contract under the influence of ATP; myosin without actin cannot contract.

In this connection it is interesting to consider some aspects of the fine structure of muscle. The electron microscope has shown that myosin in the myofibril is concentrated in disk A and actin in disc U. Fibers macerated in glycerol consist of parallel fibers of actin and myosin. According to some investigators myosin and actin are found in different fibers. However, such an arrangement of actin and myosin, in the opinion of Perry [27], is improbable, since electron microscopy and sedimentation studies have shown that chaotically distributed protein particles of actomyosin contract under the influence of ATP. It is true that we must assume that the reciprocal orientation of the particles of actin and myosin in the system is very important for the normal contracting cycle. Probably this factor plays no actual role in the degree of contraction of the model fibers, since, in the absence of a load, fibers of natural actomyosin (which contain myosin B) and synthetic actomyosin fibers contract in almost the same way [13]. On the other hand, the more chaotic the distribution of myosin and actin particles with relation to each other (that is, the less the reciprocal orientation of the particles of these proteins), the less the tension and resistance to breaking of the model.

As we observed, monolayer fibers of actomyosin are characterized by a large modulus of elasticity and a high capacity for resistance to breaking, but their strength and the tensions which they develop are lower than in muscle.

The monolayer fibers contract much more slowly than muscle. Thus, a monolayer fiber shortens 40% in 2 min , while in a cross-striated muscle the contraction lasts,altogether,0.1 sec., that is, the muscle contracts 1200 times faster than the monolayer fiber. This difference in rate of shortening is produced as follows: 1) in the monolayer fiber, in spite of its definite structure, the natural orientation of the actin and myosin found in the live muscle is destroyed; 2) the rate of splitting of ATP is limited by the diffusion of the latter into the fiber; 3) it is possible that the energy for the mechanical work of the muscle comes not only from ATP, but also from some other energy source. This problem has been considered in the communications [27-30,34].

The importance of actin for muscle activity has been further shown by some studies of a comparative biochemical and embryological biochemical character. Comparative study of the actomyosin of skeletal, cardiac and smooth muscle showed that the bond between actin and myosin is strongest in actomyosin of the uterus and weakest in the actomyosin of cardiac muscle [31]. It has also been shown that on the rapidly contracting flight muscle of insects the bond between myosin and actin is much weaker than in the slowly contracting femoral muscle [32]. Thus, the strength of the bond between actin and myosin in actomyosin of different muscles decreases with increased rate of contraction. The addition to actomyosin of actin removed from the flight muscle causes an increase in viscosity, which indicates the presence in this actomyosin of free myosin.

As a result of the investigations of Hayashi and co-workers [21], it has been established that the tension developed by the monolayer fibers depends on the ratio of actin and myosin in actomyosin. These results agree fully with our data, from which it follows that the maximum shortening (50%) of the monolayer fibers is developed when the content of actin in the actomyosin complex is 27%. If the picture obtained with the model fibers is close to the one found in vivo, then we can assume that the different shortening powers shown by tonic and tetanic muscles are completely determined by the ratio of actin and myosin in the actomyosin of these muscles.

The investigations of Ebert et al. [33] are of considerable interest. They showed by the use of a serological method that in the chick embryo the first beat of the heart is not found if actin is blocked by the corresponding antiserum. It is clear that the blocked actin cannot already have been combined with myosin to form the actomyosin complex. We can conclude from this that contraction is exclusively a property of actomyosin, a complex which has new properties arising from the combination of actin with myosin. This is a functionally unique system which combines in itself enzymatic and contractile functions.

It is known that ATP causes reversible contraction of ameba, model cells (fibroblasts), and also spontaneous movement of spermatozoa, rhythmic motion of flagella, etc. Clearly, the mechanical effect is caused by the reaction of their proteins with ATP. The actomyosin-like substance obtained from the plasmodium of myxomycetes, like actomyosin, changes its properties when ATP is added [34]. Thus, the theory of Engel'gardt

of the "actor–catalyst" becomes still more firmly grounded as new facts accumulate. Evidently one and the same process lies at the basis of all motile reactions of living organisms, namely, the reaction of an actor-catalyst with ATP. Muscle actomyosin, as compared to the contractile apparatuses described above, is a more highly organized contracting complex, consisting of two proteins, myosin and actin. Evidently, in the evolutionary development of the animal kingdom there has also been evolution of the contracting substance. As a result of this evolution the structural proteins (actin and myosin) and the enzymes (adenosinetriphosphatase, creatinephosphorylase, etc.) which are needed for contraction have been concentrated in the contracting substance.

SUMMARY

Monolayer threads of myosin and actomyosin are elastic-viscous systems with a complex spatial structure. The modulus of elasticity of the actomyosin threads E is 14×10^{-2} kg/cm^2, while the modulus of elasticity of the myosin threads is 5.6×10^{-2} kg/cm^2. ATP decreases the modulus of elasticity of actomyosin threads (5.62×10^{-2} kg/cm^2) but does not change the elasticity of myosin threads.

Myosin monolayer threads do not contract under the action of ATP. On the contrary, at pH > 7 lengthening of the monolayer threads is observed. The contraction of the actomyosin monolayer threads under the action of 5×10^{-3} M ATP is 40-50%. The percentage of the contraction depends on the concentration of ATP. The maximum of contraction is observed when the concentration of ATP is 5×10^{-3} M. The decrease of the concentration of ATP causes a reduction of the amplitude of the contraction to 20 per cent (at 1×10^{-3} M ATP). The maximum of contraction occurs at pH 7.5-7.6.

Muscle contractility is an exceptional property of actomyosin. The latter is an integral functional system consisting of actin and myosin. The role of actin is to form adequately arranged stable structure of contractile substance, on the one hand, and of the labile complex, on the other, which can interact reversibly with ATP, accomplishing thus the normal process of contraction.

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CHANGES IN THE BIOCHEMICAL PROCESSES IN GRAPES UNDER THE INFLUENCE OF A MICROFERTILIZER WHICH CONTAINS CHROMIUM

O. K. Dobrolyubskii

Odessa Agricultural Institute

Chromium belongs to the fourth group of the Mendeleev periodic table and is very similar to such elements, well known for their biological activity, as Mn, Fe, Co, Ni, and Cu. Although each of these has a specific action, there is a definite similarity in the behavior of their compounds toward the organism. Such a similarity results from the presence in them of an unsaturated valence d-level [1] which assures a variable valence (for Cr in particular, the possible shifts are $Cr^{2+} \rightleftharpoons Cr^{3+} \rightleftharpoons Cr^{6+}$, which give to chromium compounds the power to take part in oxidation-reduction processes), and also the tendency to complex formation. This similarity is found not only in the effects of the microelements themselves, but also in the fact that they can often replace each other.

Compared to these other microelements, the biochemical behavior of Cr has been little studied [2], especially with respect to plant organisms [3]. Pirschle [4], Scharrer and Schropp [5], and Reinbold and Hausrath [6] have shown the varied effects of Cr compounds, including toxic ones, on some agricultural cultures. Recently we have shown the effects of Cr^{3+} compounds on grapes [7] and a number of other agricultural crops [8].

EXPERIMENTAL

In the experiments with grapes we used $Cr_2(SO_4)_3$ as a microfertilizer, using two concentrations: calculated at 0.005 g of salt per plant (which we called $Cr_2(SO_4)_3$-I) and 0.2 g of salt per plant ($Cr_2(SO_4)_3$-II). The microfertilizer was added during the period of blooming of the plant by the method described before [7]. The soil of the vineyard was southern black earth with a weakly alkaline reaction (pH 7.2-7.3), and which contained on the average 0.018% Cr [9].

The experiments were carried out on grapes of the Aligot species. Four experiments were run in each series. Samples were taken from not less than 20 plants of about the same development; the weight, sugar content and acidity of the grapes were determined by the methods described earlier [10]. The glucose-acidimetric index (GAI), which characterizes the ripeness of the grapes, was calculated as the ratio of amount of sugar in the grapes to their titratable acidity.

The pH value and the oxidation-reduction potential of the leaves and grapes were determined in 2 gram samples by the potentiometric method [11]. The oxidation-reduction index (rH) was calculated by the formula $rH = E_H/0.029 + pH$ (where E is the oxidation-reduction potential).

Determination of glucose, fructose, and sucrose was carried out by the method of Bertrand; the content of organic acids, and catalase and invertase activity were determined as described before [7]; the activities of ascorbic oxidase, polyphenol oxidase, and peroxidase were determined by the method of Povolotskaya and Sedenko [12].

The results given in Table 1 show that in five years the weight of 100 grapes increased corresponding to the greater or smaller amount of $Cr_2(SO_4)_3$ by 23 and 28%. $Cr_2(SO_4)_3$-II showed a more favorable effect; the sugar content reached a higher value and the titratable acidity fell more sharply than in the controls.

TABLE 1

Effect of the Microelement Cr on Weight, Sugar Content, Acidity, and GAI Value of Grapes

Item	Experimental conditions	1954	1955	1956	1957	1958	Five year average
Average weight, 100 grapes	control	114	116	130	94	108	112
	$Cr_2(SO_4)_3$—I	—	141	153	133	152	114
	$Cr_2(SO_4)_3$—I	138	132	150	119	153	138
Amount of sugar, %	control	17.3	19.0	13.58	13.5	16.95	15.92
	$Cr_2(SO_4)_3$—I	—	19.15	15.65	16.85	18.1	17.43
	$Cr_2(SO_4)_3$—II	19.6	19.1	16.6	16.3	18.1	17.92
Titratable acidity in g/liter tartaric acid	control	8.8	9.7	18.3	19.5	11.25	13.51
	$Cr_2(SO_4)_3$—I	—	9.05	16.6	13.5	9.55	12.18
	$Cr_2(SO_4)_3$—II	7.2	9.05	15.7	14.5	10.25	11.34
GAI value	control	1.96	1.95	0.75	0.69	1.50	1.37
	$Cr_2(SO_4)_3$—I	—	2.11	0.94	1.25	1.89	1.54
	$Cr_2(SO_4)_3$—II	2.72	2.11	1.06	1.12	1.76	1.75

TABLE 2

Effect of the Microelement Cr on the Oxidation-Reduction Potential (E_H), Oxidation-Reduction Index (rH) and pH Value of Leaves and Grapes

Index	Experimental conditions	Leaves, August 25	Leaves, Sept. 24	Grapes, Sept. 9
E_H in volts	control	0.457	0.579	0.763
	$Cr_2(SO_4)_3$—I	0.445	0.593	0.719
	$Cr_2(SO_4)_3$—II	0.429	—	0.719
rH	control	27.81	29.75	33.57
	$Cr_2(SO_4)_3$—I	26.16	30.05	32.25
	$Cr_2(SO_4)_3$—II	26.71	—	31.79
pH	control	6.03	4.89	3.63
	$Cr_2(SO_4)_3$—I	5.41	4.81	3.88
	$Cr_2(SO_4)_3$—II	5.61	—	3.65

TABLE 3

Effect of Microelement Cr on the Activity of Catalase in Leaves and Grapes in ml of 0.1 N H_2O_2

Experimental conditions	Object and date of analysis		
	leaves, Aug. 25	leaves, Sept. 24	grapes, Sept. 8
Control	0.30	1.02	0.35
$Cr_2(SO_4)_3$—I	2.89	3.16	1.06
$Cr_2(SO_4)_3$—II	3.38	—	1.76

The observed increased rate of ripening of the grapes was confirmed by the increased value of GAI from 1.37 to 1.54 when $Cr_2(SO_4)_3$-I was used and even to 1.75 when $Cr_2(SO_4)_3$-II was used.

More rapid ripening under the influence of chromium can be explained by the active participation of Cr^{3+} in different oxidation-reduction processes which occur in the plant. Changing the conditions of a given oxidation-reduction reaction causes a change in activity of enzymes, the photosynthetic process, etc., as a result of which hydrolytic processes predominate in the cells, the photosynthetic products dissolve as intermediate forms and pass to the point of deposition in the storage area of the plant [13].

One of the most important indications of the degree of the oxidation-reduction processes is the value of the oxidation-reduction potential (E_H) of the system.

TABLE 4

Effect of Microelement Cr on the Activity of Ascorbic Oxidase, Polyphenol Oxidase, and Peroxidase in Leaves and Grapes (activity expressed in mg ascorbic acid oxidized per 1 g tissue)

Object and date of analysis	Experimental conditions	Ascorbic oxidase		Polyphenol oxidase		Peroxidase	
		pH 5	pH 7.5	pH 5	pH 7.5	pH 5	pH 7.5
Leaves 23. VIII 57	Control	72.4	32.2	50.7	69.5	136.1	111.7
	$Cr_2(SO_4)_3$—II	59.4	48.3	70.0	83.2	59.9	81.6
Leaves 2. X 57	Control	11.3	20.3	26.8	57.9	55.6	0
	$Cr_2(SO_4)_3$—I	18.0	18.5	23.2	77.2	63.3	7.2
Leaves 25. VIII 58	Control	12.9	10.3	0	42.7	2.6	41.2
	$Cr_2(SO_4)_3$—I	17.5	14.4	27.8	54.6	25.8	—
	$Cr_2(SO_4)_3$—II	22.1	21.2	19.0	50.4	11.9	52.0
Leaves 24. IX 58	Control	22.8	17.6	31.4	40.4	59.3	85.6
	$Cr_2(SO_4)_3$—I	23.9	18.4	27.7	68.4	62.4	101.8
Grapes 2. IX 57	Control	32.5	63.2	17.5	0.8	94.5	155.3
	$Cr_2(SO_4)_3$—I	27.5	50.0	49.5	59.0	32.5	109.0
Grapes 8. IX 58	Control	24.0	31.0	11.0	19.0	63.0	72.2
	$Cr_2(SO_4)_3$—I	28.7	32.4	20.7	36.1	71.8	106.4
	$Cr_2(SO_4)_3$—II	42.1	37.7	12.7	34.9	65.9	91.7

TABLE 5

Effect of Microelement Cr on the Content of Total Sugars, Invert Sugar and Sucrose, and Activity of Invertase in Leaves and Grapes

Object and date of analysis	Experimental conditions	Total sugar, %	Invert sugar, %	Sucrose, %	Invertase activity, mg%
Leaves 25. VIII	Control	2.12	1.73	0.38	492.8
	$Cr_2(SO_4)_3$—I	2.31	1.92	0.38	504.4
	$Cr_2(SO_4)_3$—II	2.06	1.67	0.38	532.0
Leaves 24. IX	Control	2.33	1.82	0.49	486.4
	$Cr_2(SO_4)_3$—I	2.85	1.82	0.99	536.0
Leaves 8. IX	Control	12.15	12.15	Traces	385.0
	$Cr_2(SO_4)_3$—I	14.00	14.00	"	402.0
	$Cr_2(SO_4)_3$—II	13.84	13.84	"	427.1

TABLE 6

Effect of the Microelement Cr on Accumulation of Different Sugars in Grapes in %

Experimental conditions	Invert sugar	Fructose	Glucose	F/G ratio
Control (1955)	13.22	6.0	7.22	0.83
$Cr_2(SO_4)_3$—I	14.52	7.0	7.52	0.92
Control (1956)	12.10	3.87	8.23	0.47
$Cr_2(SO_4)_3$—I	13.60	4.64	8.96	0.52
Control (1957)	16.50	8.40	8.10	1.00
$Cr_2(SO_4)_3$—I	19.02	9.82	9.20	1.07

TABLE 7

Effect of Microelement Cr on Total Organic Acid Content in Leaves and Grapes

Experimental conditions	Leaves VIII		Leaves IX		Grapes IX	
	I	II	I	II	I	II
Control	45.82	34.4	49.98	37.5	36.65	27 5
$Cr_2(SO_4)_3$ -- I	55.81	41.9	54.98	41.3	48.31	36.3
$Cr_2(SO_4)_3$ —II	49.88	37.5	—	—	46.65	35.0

Note: I — in ml of 0.01 N NaOH; II — in ml tartaric acid

As a result of the use of the Cr^{3+} compound, in August there was a marked decrease in E_H in the leaves, especially in the variant with $Cr_2(SO_4)_3$-II (Table 2). Such an increased rate of the oxidation process confirmed our previous experiments in which we observed an increase in oxidizability of leaf tissues for variant $Cr_2(SO_4)_3$-I from 24.4 to 26.7 (in ml of 0.1 N $KMnO_4$). An analogous effect was observed in grapes before they reached their technical maturity (sample on September 8); the E_H value at this period was also decreased.

The E_H value depends on many factors: ionic strength of the solution, presence of complex-forming substances, and pH value. It is especially characteristic that the microelement lowers the pH value in the leaf rather sharply, from 6.03 to 5.41 and 5.61 (for variants $Cr_2(SO_4)_3$-I and $Cr_2(SO_4)_3$-II respectively). During the process of vegetation, the E_H value rises and the pH of the leaf falls. In the grapes, because of lessened titratable acidity (Table 1), the Cr^{3+} compounds raise the pH value.

In cellular processes, the oxygen-hydrogen equilibrium can be called the oxidation-reduction index (rH): the lower its value, the greater the reductive power of the system. The results of the analyses of leaves on August 25 showed a rather sharp decrease in rH values and their slight increase over a month. In the grapes there was also a considerable fall in rH values with an absolute rH value larger than in the leaves. This difference is evidently related to changes in photosynthetic activity in the leaves. Our experiments showed that Cr^{3+} raised the content of chlorophyll in the leaves and allowed its retention as the plant grew. Thus, before the crop was gathered on September 24, 1958, the amount of chlorophyll in the leaves for variant $Cr(SO_4)_3$-I was 221 mg/100 g compared to 181 mg in the leaves of the control grape vines.

The use of the microelement Cr^{3+} led to changes in catalase activity in the leaves. The results of these experiments are given in Table 3. As the table shows, catalase activity rises sharply under the action of Cr^{3+}, especially with large doses. The enzyme activity in August rose 9-11 fold, and at the end of the growth period this difference had decreased sharply. Chromium also strongly increases catalase activity in the grapes (3-5 fold).

In the occurrence of these changes, chromium certainly plays an important part in causing shifts in oxidase activity.

It is very interesting to study the activity of such oxidation-reduction enzymes as ascorbic oxidase, polyphenol oxidase, and peroxidase, for which the hydrogen acceptor is oxygen [14,15]. Our data on the effect of Cr^{3+} on these enzymes is given in Table 4. As this table shows, the microelement in almost all cases maintains the activity of ascorbic oxidase, polyphenol oxidase, and peroxidase at a higher level, and also permits retention of the activity of these enzymes over a longer interval of time.

The results which we obtained showed that the activities of these enzymes in an acid medium (pH 5) and a weakly alkaline medium (pH 7.5) differ greatly from each other. At the same time, the activity of the polyphenol oxidase in leaves when chromium is used was very much higher at both pH values, except at the period of the end of growth of the grapes in the $Cr_2(SO_4)_3$-I variant. The activity of the polyphenol oxidase increased especially sharply in the grapes.

There is no increased activity of peroxidase in the leaves (before the harvest) and in the grapes in the 1957 experiments under the influence of Cr^{3+}, but in the 1958 experiments in all cases we found an increase in

activity of this enzyme. Under the influence of the microfertilizer, the carbohydrate metabolism also changed. The content of total sugars in the leaves under the influence of $Cr_2(SO_4)_3$-I increased in some cases (Table 5, sample of Aug. 25, 1958), and this increase occurred only due to invert sugar ($Cr_2(SO_4)_3$-II showed no great effect in this case), and the amount of sucrose in the leaves was not changed. Before the harvest of September 24, $Cr_2(SO_4)_3$-I caused a very great rise in total sugars in the leaves, and the increase was entirely due to sucrose. In the grapes the content of invert sugar increased under the influence of the microelement (sucrose occurred only in traces in the grapes).

This effect of the microfertilizer depends to a considerable degree on the activity of the enzyme invertase, which splits sucrose into glucose (G) and fructose (F). The data of Table 5 clearly show that Cr^{3+} compounds increase the activity of invertase in leaves and grapes. Since the optimum activity of the enzyme occurs at pH 4.5, this change in invertase activity should evidently occur because the microelement lowers the pH toward this optimum (Table 2).

In Table 6 we give data on the change in accumulation of different sugars in grapes under the influence of chromium.

In spite of the certainly different meteorological conditions from one year to another, there are definite regularities in the accumulation of invert sugar, F, and G. The increase in amount of invert sugar is not accompanied by a proportional increase of F and G. With ripening of the grapes there was a gradual transformation of G→F, as a result of which the ratio F:G approached 1 and then somewhat surpassed it. As Table 6 shows $Cr_2(SO_4)_3$-I in all cases increased this ratio, thus permitting an increased rate of ripening of the grapes.

Under the influence of the microelement, the dynamics of the accumulation of organic acids also changed in the leaves and grapes. The difference in content of organic acids is due to the different in relation of the rate of enzymatic reactions which lie at the basis of formation and transformation of the complex organic acids. Besides the total titratable acidity in the grapes (whose results were given in Table 1), we studied the content of the sum of all the organic acids, free or bound, in leaves or grapes. These data are given in Table 7.

In both variants of the dose of microfertilizer, especially in the experiments with $Cr_2(SO_4)_3$-I, we observed an accumulation of total organic acids in the leaves and grapes. Since in the grapes the content of free acid is always decreased under the influence of the microelement (Table 1), and the sum of all the organic acids is increased (Table 7), we can assume the accumulation in the grapes of so-called bound acid, which most often occurs in plants in the form of calcium and potassium salts.

SUMMARY

The use of microfertilizers which contain Cr in raising grapes can greatly increase the weight of the grapes and their sugar content, can lower the titratable acidity, and can hasten the ripening. The microelement changes the value of the oxidation-reduction potential (E_H), the oxidation-reduction index (rH), and the pH, and increases the activity of catalase, ascorbic oxidase, polyphenol oxidase, peroxidase, and invertase. More chlorophyll accumulates in the leaves; the total organic acid content increases in the leaves and grapes, the sugar content changes, and in the grapes there is an increase in amount of glucose and especially of fructose.

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METABOLISM IN TISSUE EXPLANTS OF THE MAITLAND TYPE

O. N. Panchenko, V. S. Samsonova, and I. I. Khripunova

Culturing peices of tissue in vitro in physiological saline which contained glucose and serum was first carried out in 1928 by Maitland [1] who raised virus vaccine in this medium. Later this type of tissue explant was widely used in practical virusology because of the great simplicity of its preparation; it was used for culturing different viruses and rickettsia outside the host organism [2-4]. However, the biochemistry of this medium has not yet been studied if we do not accept as accurate the work of Zinsser and Schoenbach which they published in 1937 [5].

In the present paper we give the results of a study of the metabolism of tissue explants of the Maitland type; we give the results of the determination of the intensity of respiration and anaerobic glycolysis in pieces of chick embryo tissue while it is cultured in vitro without renewal of the nutritive medium. We also give data on change in chemical composition of the medium and of the explants themselves during their culturing.

MATERIAL AND METHODS

The object of study was 11-day-old tissue of chick embryo, finely minced with scissors, placed in a Tyrode-serum mixture with the ratio 200 mg of tissue to 3 ml of medium. The latter consisted of 3 parts Tyrode glucose-salt solution and 1 part horse serum. Incubation was carried out under sterile conditions in a 50 ml Erlenmeyer flask, hermetically sealed with a resin stopper at 37° for 10-20 days. At 1, 3, 5, 7, 10, and 15-day intervals we took from the whole collection of samples (100-200 flasks) of tissue explants 4-5 flasks, whose contents were combined and filtered; the precipitate, which consisted of pieces of tissue, was washed three times with physiological saline and partly dried by sucking air over it with a water pump. On 100 mg samples we determined the intensity of respiration and glycolysis manometrically in a Warburg apparatus. The results were calculated on dry weight. In a number of experiments the viability of the cultured tissues was controlled by transplanting the tissue pieces to a Carrell vessel [6].

The content of the various substances in the nutritive medium and in the cultured tissue was determined respectively in the filtrate and in the washed precipitate of tissue explant of a given age. Glucose was determined according to Hagedorn-Jensen, glycogen according to Genkin [7] and Seifter [8] in our modification, which allowed us to determine glycogen in tissue with a low content. In this method the tissue under study was boiled for 45 min in 30% KOH and from the resulting protein solution the glycogen was twice precipitated with double the volume of alcohol in the presence of a salt (10% Na_2SO_4). The copious glycogen-salt precipitate dissolved easily in hot water with the formation of a perfectly clear solution in which the glycogen content was determined colorimetrically by the anthrone reaction [8]; inorganic phosphorus was determined according to Taussky [9], protein in the medium by the Kjeldahl method and nephelometry [10], protein in the tissue by the gravimetric method of Winnick [11], amino nitrogen according to Van Slyke, ammonia according to Sachkov [12]; carbon dioxide in the gas phase was determined by titration of a 0.1 N solution of $Ba(OH)_2$ which has absorbed the carbon dioxide removed from the gas phase of the tissue culture by a stream of air free from CO_2, lactic acid by its oxidation to acetaldehyde and later iodometric titration [13]; pH was determined colorimetrically by visual comparison with the corresponding standard phosphate buffers which contained phenol red as indicator.

Effect of Yeast on the Reducing Activity of the Culture Medium of Tissue Explants of Different Ages (in mg%). Experimental conditions: to 1.25 ml of culture filtrate added 0.25 ml of fresh yeast suspension (1:2). Incubation carried out at room temperature for 45 min. Samples with boiled yeast suspensions served as controls for reducing substances contained in yeast

Sample	Age of culture in days				
	0	1	3	7	10
Culture medium	94	26	2	37	55
Boiled yeast	34	16	17	12	15
Medium + boiled yeast	126	37	16	46	71
Medium + fresh yeast	32	14	16	49	71

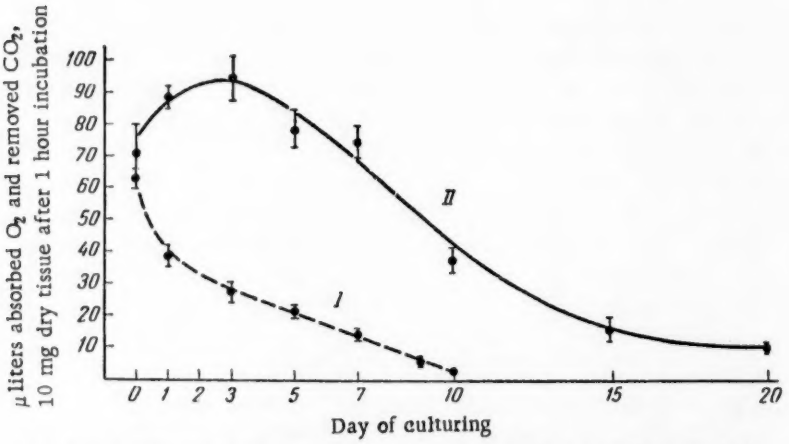

Fig. 1. Change in intensity of respiration and anaerobic glycolysis in chick embryo tissue during its culturing in vitro. Experiment conditions: 1) gas medium (O_2), 100 mg of wet tissue placed in 1.9 ml of Ringer-phosphate buffer (pH 7.3-7.4), in a central vessel 0.5 ml of 10% KOH, incubation at 37° for 1 hour; 2) gas medium (mixture of N_2 and CO_2), 100 ml of wet tissue placed in 1.9 ml of Ringer-carbonate buffer (pH 7.3-7.4), in central vessel 0.5 ml H_2O, incubation at 37° for 1 hour. Percentage error of arithmetical mean (p) in determination of glycolysis in 15 days > 0.1 and < 1.0, in 20-5.0. In the determination of respiration in 9 days p > 1.0 and < 5.0. At all other stages of the determinations p < 0.1.

All the numerical data obtained in this work* were treated statistically [14] and presented in the form of curves in which we have given a graphical representation of the variation of the arithmetical average from the general mean. The percentage error of the determinations is given in the notes to the figures.

RESULTS

Respiration and Glycolysis in Pieces of Chick-Embryo Tissue Cultured In Vitro according to Maitland

During culturing of the chick embryo tissue there was a progressive fall in intensity of absorption of oxygen by the tissue from 60 μ liters of O_2 at the beginning of culturing to 3-5 μ liters O_2 on the 10-15th day. The

*Except the data given in the table, which was not submitted to statistical treatment and which did not require it.

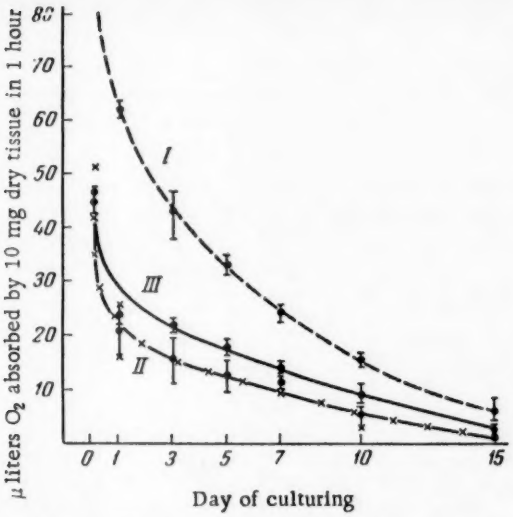

Fig. 2. Effect of glucose and succinic acid on intensity of respiration of tissue explants. Experimental conditions the same as in Fig. 1. 1) Experiment with addition of succinate; after 3, 5 7, and 10 days $p > 0.1$ and < 1.0; 2) experiment with addition of glucose; after 1, 3, 5, and 10 days, $p > 0.1$ and < 1.0; 3) control after 10 days $p > 1.0$ and < 5.0. At all other stages of the determinations (for all experiments), $p < 0.1$ except at 15 days when $p \approx 5.0$ (in all experiments).

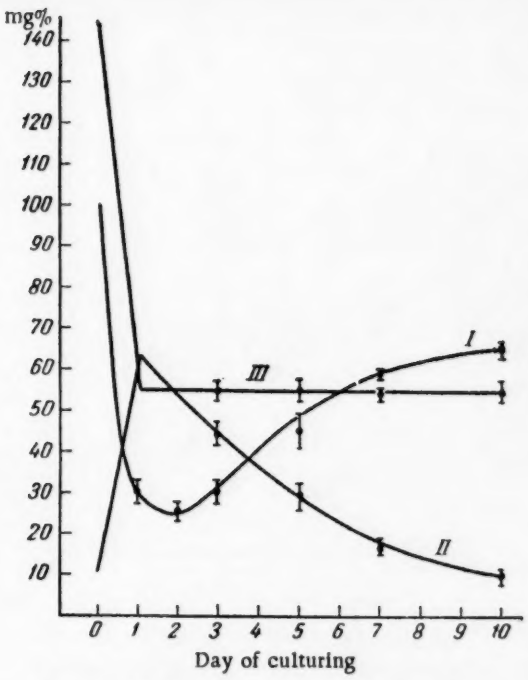

Fig. 3. Change in content of reducing substances, lactic acid, and glycogen during culturing of the tissue. I) Reducing substances of the nutritive medium; II) lactic acid of the nutritive medium; III) glycogen of the tissue. In all cases $p > 1.0$ and < 5.0; values which predominate are $p > 0.1$ but < 1.0; most of the determinations were made with values of $p < 0.1$.

sharpest fall in intensity of respiration occurred on the first day of culturing (Fig. 1))

The addition of succinic acid to the sample (final concentration 0.05 M) had no effect on the character of the change in intensity of respiration of the tissue during culturing, but raised the total level of oxygen absorption by the cultured tissue, and in the same degree, fresh tissue also (Fig. 2). On the other hand, the addition of glucose (final concentration 200 mg%) (Fig. 2) greatly increased the intensity of respiration in the tissue explants, but at the same time it did not have an effect on fresh embryonic tissue. Fig. 1 shows that the intensity of anaerobic glycolysis in pieces of chick embryo tissue on the first day of culturing rises markedly, and then, beginning on the 5-7th day, falls rather sharply. However, even on the 20th day of culturing, the glycolytic activity of the explants can still be determined in definite amounts (≈ 10 μ liters CO_2).

Change in Chemical Composition of the Medium and Tissue During Process of Culturing

Change in Content of Components Which Characterize Carbohydrate Metabolism

During the first day of culturing (Fig. 3, curve I), the content of glucose in the medium falls to 25-30% of the starting level. From about the third day the amount of reducing substances in the culture medium begins to rise gradually, reaching on the 10th day 60% of the starting level. Preliminary treatment of the culture medium with yeast shows (Table) that the reducing substances which appear in the medium in the later stages of culturing are not fermented by yeast, that is, are substances of a nonglucose nature.

The intense splitting of glucose which we have observed in the culture medium under conditions of free

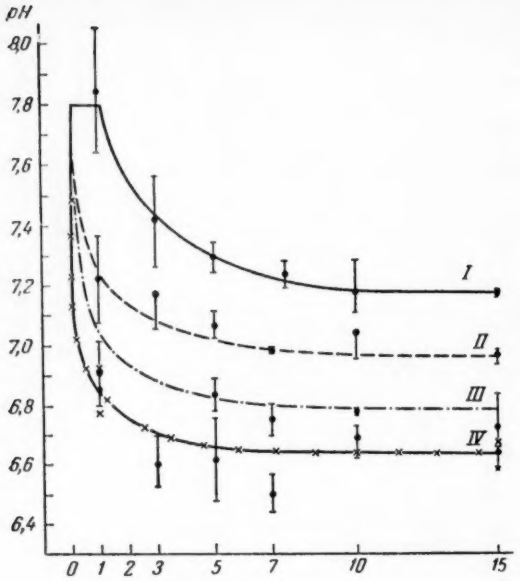

Fig. 4. Change in pH value of the culture medium depending on glucose content. I) 1.0 mg% glucose in the medium; II) 100.0 mg% glucose in the medium; III) 300.0 mg% glucose in the medium; IV) 400.0 mg% glucose in the medium. In three cases $p > 0.1$ and < 1.0; in all the other stages of culturing (all series of experiments) $p < 0.1$.

access of air (aerobic glycolysis) is accompanied by the appearance in the medium of lactic acid (Fig. 3, curve II). However, in spite of our expectations, from the first day of culturing the content of lactic acid in the medium begins to fall (from 60 to 10 mg%), although the intensity of glycolysis after the first day of culturing continues to increase (Fig. 1). Thus, under the conditions of our study, the Maitland tissue system does not accumulate lactic acid in the medium, but causes it to undergo some further transformation.

As Fig. 3, curve III shows, after the first day of culturing there is a sharp decrease in amount of glycogen in the tissue, while on later days of culturing, the content of glycogen in the tissue no longer changes but remains on the average equal to 55-60 mg%.

Change of Reaction of the Medium During Culturing

Fig. 4, curve II characterizes the change of pH in the nutritive medium with the content of glucose usually used (100 mg%). As the figure shows, the pH of this medium falls most sharply (from 7.8 to 7.1) during the first three days, while on all the later 12 days the fall in pH is only 0.1-0.2.

The value of pH depends considerably on the

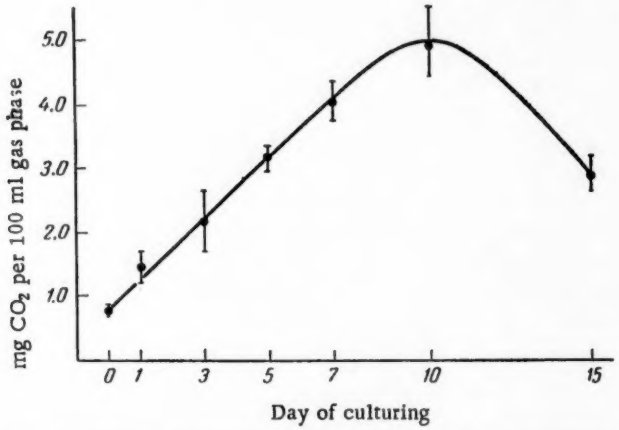

Fig. 5. Change in content of carbon dioxide in the gas phase. On the 1st and 7th day of culturing $p < 1.0$ and > 0.1; on the 3rd, 10th, and 15th day of culturing $p > 1.0$ and < 5.0; before incubation and on the 5th day of culturing, $p < 0.1$.

initial glucose content in the nutritive medium: the higher this is, the lower is the pH value at all stages of culturing. However, in the complete absence of glucose in the culture medium during culturing, there is a considerable fall in pH, occurring in general in the same way as in media which contain glucose (Fig. 4). The

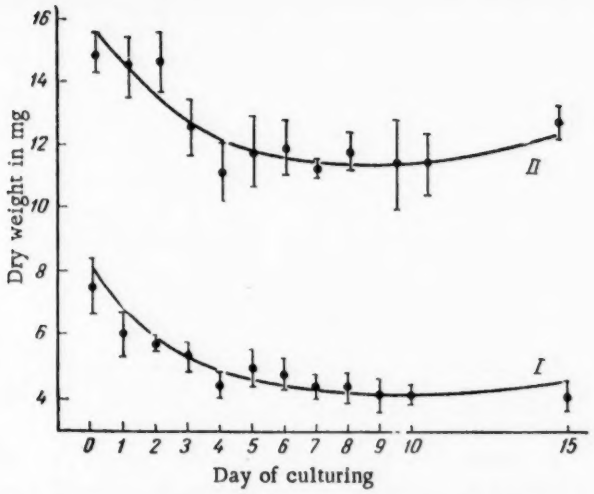

Fig. 6. Change in weight of protein (I) and tissue (II) in samples of tissue explants during incubation. In determinations of weight of tissue in all stages of culturing $p < 0.1$; in determination of weight of protein at the beginning of incubation (0) and on the 1, 2, 4th day, $p < 0.1$; on 3, 5, and 8th day, $p > 0.1$ and < 1.0, and on 6,7,9,10 and 15th day of culturing, $p \approx 1.0$.

relative stabilization of pH of the nutritive medium noted after the third day of culturing is evidently due to the removal of the constantly formed carbon dioxide from the liquid phase into the gas phase of the biological system which we study. This regularity is illustrated in Fig. 5, which shows that up to ten days the CO_2 content in the gas phase steadily rises.

Some Indications of Intensity of Autolysis in the Studied Systems of Tissue Explants

According to the data given in Fig. 6, on the 15th day of incubation the amount of protein in the tissue explant has decreased on the average from 7.5 to 4.2 mg per sample, and the quantitative change in protein goes practically parallel to the change in weight of the whole tissue. As a result of this parallelism, the protein content in the cultured tissue remains almost constant (40.0%) during the whole incubation period. When we consider the curves given in Fig. 6,we see that the sharpest autolytic change in the tissue explants occurs during the first three days; then a period of some stabilization follows.

We can form some estimate of the size of the autolysis in the system which we study from the data on accumulation of nonprotein nitrogen in the culture medium and also of inorganic phosphorus, as shown in Fig. After 10 days of culturing, with a relatively constant content of protein nitrogen, the content of nonproteinN in the medium rises to about 30 mg%; in this figure the content of amino nitrogen increases to about 8 mg% and of ammonia nitrogen to 4 mg%. We should also relate the accumulation of inorganic phosphorus (up to 5 mg%) to this same autolytic process; this occurs chiefly on the first day of culturing.

DISCUSSION OF RESULTS

The object of our study is a complex biological system in which there are present,simultaneously,three tyoes of cells of different tissues: surviving, degenerating, and proliferating. Around the pieces of tissue attached to the bottom of the flask we observed macroscopically and microscopically a wide zone of cell growth. In view of the nonhomogeneity of the system which we studied, the data given in this work must be considered as integrating a very complex and varied set of processes,which makes their interpretation difficult. Therefore, in discussing the results, we abstain from any explanation of the reasons for our findings and limit ourselves only

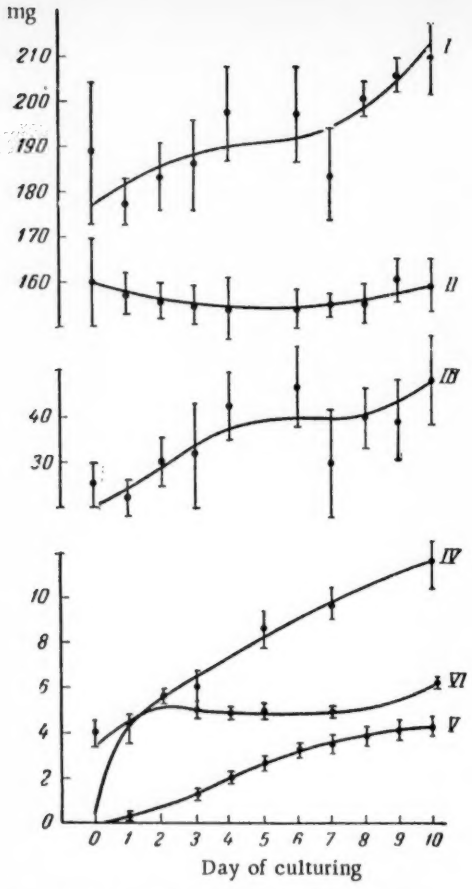

Fig. 7. Change in content of the different nitrogen fractions and inorganic phosphorus in the medium during culturing. I) total nitrogen; II) protein nitrogen; III) nonprotein nitrogen; IV) amino nitrogen; V) ammonia nitrogen; VI) inorganic phosphorus. In all cases $p > 1.0$ and < 5.0; predominant value $p > 0.1$ but < 1.0; most of the determination were made with a value of $p < 0.1$.

to a short generalization of those effects which are characteristic in the metabolism of systems suitable for raising viruses and rickettsia.

We should chiefly say that in our biological system the processes of tissue breakdown prevail quantitatively over processes of synthesis, especially in the first three days of culturing. This is indicated not only by the loss of tissue and the accumulation in the medium of products of breakdown with a low molecular weight, but also by the sharp fall in intensity of the respiratory processes during the first days of culturing. At the same time, the intensity of glycolysis of the cultured tissue is increased in this period very sharply, remaining above the intensity of glycolysis of the initial fresh embryonic tissue for 6-7 days of culturing.

The high activity of the glycolytic processes in the studied system of tissue explants is also indicated by the rapid disappearance from the nutritive medium of glucose, the appearance in it of lactic acid, and the mobilization of 2/3 of the tissue glycogen. This special characteristic of the carbohydrate metabolism of the studied system is also shown by the conditions in it for destruction of lactic acid and the accumulation of reducing substances of a nonglucose character.

SUMMARY

During incubation of pieces of chick-embryo tissue in Tyrode-serum mixture, there is a progressive fall in intensity of endogenous respiration from 60 μ liters of oxygen before beginning the incubation to 3-5 μ liters of oxygen on the 10-15th day. The sharpest fall in intensity of respiration (to 20 μ liters) occurs on the first day of culturing.

Succinic acid added to the incubation medium stimulates the intensity of tissue respiration of the transplants (as also the respiration of the initial chick embryo tissue) at all stages of culturing to the same extent. Glucose under the same conditions does not show any great effect on the intensity of respiration of the initail embryonic tissue, but reduces(by 30-40%) the respiration of this tissue cultured in vitro. In pieces of chick-embryo tissue cultured according to Maitland the intensity of anaerobic glycolysis rises during the first day of incubation and on the 3-5th day reaches a maximum (100 μ liters CO_2). From the 6-7th day of incubation there begins a rather sharp fall in the intensity of anaerobic glycolysis, which, however, still has a definite value (10 μ liters CO_2) even after 20 days of culturing.

The content of glucose in the nutrient medium of the tissue explants of the Maitland type, even in the first day of culturing, falls by 70-75%. The increase in reduction observed from the 3-4th day of incubation occurs because of accumulation in the culture medium of some sort of reducing substance of a nonglucose nature. The content of lactic acid in the culture medium reaches its greatest value at the end of the first day (60-70 mg%) and then begins to fall, reaching on the 10th day $\approx$ 10 mg%.

The glycogen content in the cultured tissue falls about threefold on the first day of incubation (from 144 to 52 mg%), then on later days of culturing its amount remains practically constant ($\approx$55 mg%).

The intensity of fall of pH during culturing is increased with increase of glucose concentration in the me-

dium. The sharpest fall in pH occurs on the first day of culturing, from 7.8 to 7.2 for a medium which contains 100 mg% of glucose, while on all the following days of incubation, the pH falls by only 0.2-0.3 altogether.

The content of CO_2 in the gas phase of the studied system of tissue explants increases evenly during 10 days from 0.7 to 5 mg/100 ml of gas phase. Then begins a sharp fall in CO_2 concentration in the gas phase.

During ten-day culturing of the tissue explants of the Maitland type, there is intense autolytic splitting of the cultured tissue as is indicated by: 1) decrease (by 45%) in weight of tissue and tissue protein; 2) increase of nonprotein nitrogen in the medium; (to 30 mg%), along with practically no change in content of protein nitrogen during culturing; 3) accumulation of ammonia nitrogen in the medium (up to 5 mg%), and of amino nitrogen to 12 mg% and of inorganic phosphorus to 5 mg%.

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THE PROBLEM OF THE ENZYMATIC ACTIVITY OF CATALASE IN A MONOLAYER

B. V. Moiseenko

Faculty of Biology and Soil Science, M. V. Lomonosov State University, Moscow

Beginning with the work of Langmuir, the intensive study of protein films which form at the boundary of phase separations has greatly enriched our ideas of the structure and physicochemical properties of protein monolayers. Although the mechanism and nature of the molecular effects when protein monolayers are formed is still not sufficiently clear, the existing information allows us to assume that at phase separation boundaries there occurs considerable deformation of protein molecules under the influence of surface forces and this is accompanied by unfolding and definite orientation of the polypeptide bonds of the protein globule.

Thus, when a protein monolayer is formed there is a change in the specific configuration of the native protein molecule in solution. Therefore the study of the biological activity of monolayers of enzymes and other active proteins is of interest from the point of view of explaining the relation between specific properties and the internal structure of the native protein molecule.

Also, a study of the biological activity of enzymes in monolayers can solve some fundamental questions of the theory of action of enzymes in the cell. The numerous separate surfaces formed by the characteristic microheterogeneity of the cell structure have an important effect on the direction and course of the processes which occur within the cell. Evidently the inhibition of the majority of processes which occur in cells takes place at the surface of the cell divisions. Combined with this is the idea that enzymes are either adsorbed on these surfaces [1-3] or spread out in monolayers [4-6]. A study of enzyme activity in monolayers can help to solve the question of which of these ideas is more accurate.

It is quite probable that the biological activity in a monolayer is retained if this specific property is connected only with a prosthetic group and the latter with its existing bond with the protein is retained in the monolayer; or, as a second possibility, if the activity is connected with the participation of peptide links which are not changed as the protein molecule spreads. However, if the enzyme owes its activity to a number of centers, reciprocally oriented on the surface of the protein globule, then it is unlikely that the activity will be kept when the monolayer is formed.

The purpose of our investigation is to study the changes in activity of enzymes when monolayers are formed.

The study of enzyme activity in a monolayer has been carried out in the work of a number of authors [7-14], but the results which have been obtained have a contradictory character which is apparently connected, on the one hand, with individual characteristics of the different enzymes, and on the other, entirely with difficulties of method. It is important in getting the desired results when studying the biological properties of protein monolayers to have the films free from even traces of materials which do not spread. This point has not received sufficient attention in a number of studies.

As the object of study in the present work we chose the enzyme catalase, since for this enzyme a prosthetic group of a hematin nature is known, and the study of its activity in a monolayer will permit us to explain the significance of the protein components, and especially their structure, for the specific activity of this protein.

TABLE 1

Determination of Enzymatic Activity of Catalase Monolayer on a Phosphate Buffer, pH 6.0. Results of titration by 0.005 N $KMnO_4$, ml

Time from beginning of expt. min	Flask	Cuvette	Difference, activity of monolayer)
0	7.4	7.4	
5	5.8	5.7	0.1
10	5.2	5.1	0.1
15	3.9	4.0	−0.1
20	3.3	3.3	0
30	2.2	2.3	0

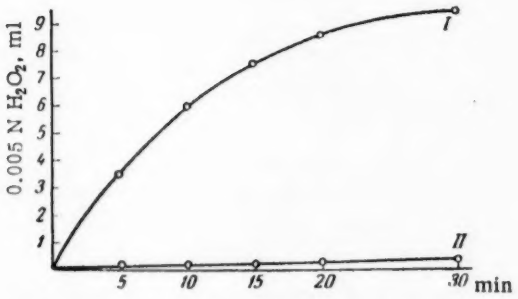

Fig. 1. Curve of decomposition of H_2O_2 by equal amounts of catalase in solution (I) and in the monolayer (II). Solution and underlayer, phosphate buffer, pH 6.0.

TABLE 2

Determination of Enzymatic Activity of Monolayer of Catalase after Washing the Underlayer with Two Liters of Phosphate Buffer, pH 6.0. Results of titration with 0.005 N $KMnO_4$, ml

Time from beginning of expt. min	Flask	Cuvette	Difference (activity of monolayer)
0	9.7	9.7	
5	9.6	9.4	0.2
10	9.5	9.2	0.3
15	9.3	8.9	0.4
20	9.2	8.7	0.5
30	9.1	8.3	0.8

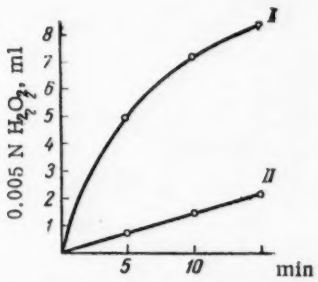

Fig. 2. Curve of decomposition of H_2O_2 by different amounts of catalase in solution (I) and in the monolayer (II). Solution and underlayer, 40% $(NH_4)_2SO_4$.

The best existing results for catalase have been obtained in the work of Kaplan [6,10] who studied the activity of catalase fibers obtained by compressing monolayers. Kaplan found that catalase fibers retained 1/5 the activity of the native enzyme. On the basis of his results, this author assumed that the catalase molecule, spread in a monolayer at an air-water interface, kept its enzymatic activity, and that denaturation of the protein, shown by insolubility and other physicochemical changes of the native protein structure did not have to lead to loss of its biological activity. On the basis of his observations both of the properties of catalase fibers and of catalase in erythrocyte cells, Kaplan concluded that the catalase molecule within the cell occurred in an insoluble state, spread on the phase separation boundaries.

The concrete problem of our work is a detailed study of the biological activity of catalase in a monolayer and to establish the true nature of this effect.

MATERIALS AND METHODS

In this work we used a preparation of crystalline catalase obtained from beef liver by the method of Summer and Dounce [15]. The catalase monolayer was obtained on the surface of a Langmuir cuvette with dimensions 75x13x1.5 cm. As the liquid we used a phosphate buffer 1/150 M, pH 6.0 and pH 6.8, and in one series of experiments, a 40% solution of ammonium sulfate. All the solutions were prepared with twice-distilled water. The activity of the catalase was determined by the method of Euler and Josephson [16]. In order to make the comparison easier between the activities of monolayers of catalase obtained under different conditions, we collected the amount of enzyme deposited on the surface in such a way that the area in all cases was the same.

For measurement of the area of the monolayer we determined the boundaries of the film with a barrier and talc. Determination of the relation of area of the monolayer to time, protein concentration, and pH of the liquid showed that this simplified method of measuring the area of the monolayer was sufficiently sensitive and permitted detection of slight changes in area under the influence of different conditions. The area of the monolayer in all the experiments was about 850 cm^2.

Determination of Catalase Activity in a Monolayer

To determine the catalase activity in a monolayer we used the following method. The substrate (in this case H_2O_2) was placed in the liquid under the monolayer and evenly mixed through the whole volume of liquid. A sample was removed directly from the cuvette to measure the activity. This method permitted a study of the activity of the monolayer without destroying its structure. The activity of the monolayer was estimated by the difference between the activity observed in the cuvette (judged by the amount of H_2O_2 which disappeared) and the activity of the underlying layer (decomposition of H_2O_2 due to molecules of catalase which touched this layer when the monolayer was formed). In order to estimate the activity of the underlying layer, after the H_2O_2 had been evenly mixed in the cuvette for 5 min , we removed from the cuvette a 50 ml sample, which served as a control; it was placed in a flask. At 5 min intervals we studied parallel samples from the cuvette and the flask. Preliminary determinations showed that during the time of the experiment (30 min), nonenzymatic decomposition of the H_2O_2 in the cuvette did not occur.

In Table 1 we give the results of one experiment on measuring the activity of a monolayer of catalase on a phosphate buffer, pH 6.0. As the table shows, the activity of underlying layer + monolayer (column "cuvette") does not differ from the activity of the underlying layer itself (column "flask"), that is, the monolayer in itself has no activity.

Consideration of the rapid decrease in amount of H_2O_2 in the flask shows, evidently, that a considerable part of the catalase deposited on the surface goes into the underlying solution. This fact is not unexpected for those conditions in which the monolayer is obtained.

Assuming that it was possible that a small activity of the monolayer of catalase might not be observed because of a high activity in the underlying layer, we tried washing the underlying layer with phosphate buffer, pH 6.0. Washing was carried out as follows: from one side of the cuvette the underlying solution was removed through a siphon and at the same time a fresh buffer was introduced into the other side of the cuvette. This method allowed us to change the underlying solution with the native molecules of catalase dissolved in it without destroying the structure of the monolayer.

As Table 2 shows, with washing, there is a considerable decrease in activity of the underlayer, and some activity of the monolayer is observed. In calculation on the basis of the volume of liquid in the cuvette (1 liter) the difference between the activity of the cuvette and underlayer averages 356 mmoles of H_2O_2 after 30 min. In order to exclude the activity of the catalase adsorbed on the stirrer, the bottom, and the walls of the cuvette, we carried out control experiments in which, before addition of H_2O_2 to the underlayer, the monolayer of catalase was removed from the surface. We found that the resulting activity corresponded to a decomposition of 213 mmoles of H_2O_2, that is, only 143 mmoles of H_2O_2 could be related to the activity of the monolayer itself. The determination showed that the activity of the monolayer was only 1-3% of the activity of equal amounts of native catalase in solution (Fig. 1).

Nature of the Residual Activity of the Monolayer

The slight residual activity of the monolayer which was observed could be explained by a small amount of native molecules of catalase adsorbed on the monolayer. If this is so, then the residual activity of the monolayer should be higher, the better the conditions for adsorption, and vice versa.

To test this idea, we ran experiments with an underlayer of phosphate buffer, pH 6.8, in which the solubility of catalase is higher than in the buffer with pH 6.0, and in 40% ammonium sulfate solution, in which the solubility of the protein is minimal. Measurement of the activity of the monolayer of catalase in 40% $(NH_4)_2SO_4$ solution showed that in this case the activity of the underlayer was zero, since, because of the insolubility of the catalase, it did not pass from the surface into the underlayer. The activity of the monolayer was higher (712 mmoles H_2O_2 after 15 min) than in the experiments with a phosphate buffer, pH 6.0, at the same area of monolayer.

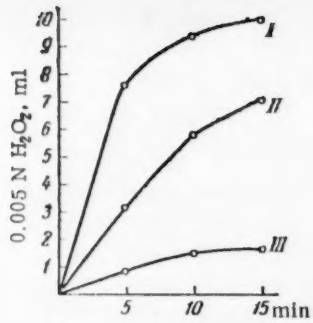

Fig. 3. Effect of composition and pH on the activity of catalase fibers. I) underlying solution, 40% $(NH_4)_2SO_4$; II) underlying solution, phosphate buffer, pH 6.0; III) underlying solution, phosphate buffer, pH 6.8.

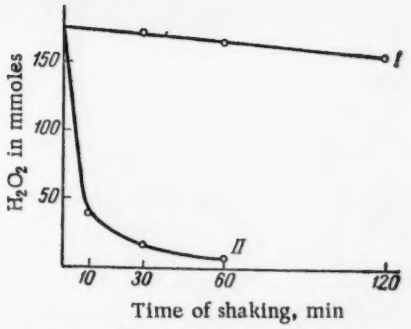

Fig. 4. Effect of pH on rate of inactivation of catalase solutions with shaking. I) pH of solution 6.8; II) pH of solution 6.0. Time of shaking the catalase solution on abscissa; amount of H_2O_2, in mmoles, decomposed in the sample after 5 min on the ordinate.

When the same amount of catalase was added to an equal volume of underlying 40% $(NH_4)_2SO_4$, we found that the catalase activity of such a mixture was about four times higher than the activity of the catalase in the monolayer (Fig. 2). Evidently when the catalase formed the monolayer, it lost about 75% of its activity. To explain the reasons for the residual activity of the catalase monolayer in these experiments we ran the underlayer with a phosphate buffer of pH 6.8. The activity of the monolayer then fell sharply (280 mmoles H_2O_2 against 712 mmoles H_2O_2). This indicates that the activity of the catalase monolayer depended on adsorbed native catalase molecules which were desorbed when the underlayer was separated from the monolayer. However, we also noted that the observed lower activity (280 mmoles H_2O_2) was not the activity of the monolayer itself.

As specially run experiments showed, a considerable part of the activity (190 mmoles H_2O_2) depended on native molecules adsorbed on the stirrer, bottom, and sides of the cuvette, and the rest (90 mmoles H_2O_2) of the residual activity was evidently connected with molecules adsorbed on the monolayer and not removed at the time of washing with phosphate buffer. Measurement of the activity of the catalase monolayer obtained on a phosphate buffer, pH 6.8, did not show any difference between the activity in the cuvette and in the underlying layer even after washing the latter. This is because the good solubility of catalase in the underlying layer at pH 6.8 reduces the adsorption of the native molecules on the stirrer, bottom and walls and monolayer almost to zero. The absence of a difference between activity in the cuvette and the underlying layer both before and after washing the underlying layer shows that the monolayer itself does not have activity.

Thus, the experiments run under different conditions of measurement of enzymatic activity of the catalase monolayer with a comparatively large area give the same answer: the transfer of catalase molecules into the monolayer is accompanied by complete loss of enzymatic activity, while the observed activity of the monolayer depends on the native catalase molecules adsorbed on it.

Determination of Enzyme Activity of Catalase Fibers

The data which we obtained on the loss of activity of the monolayer raises the question of the observations of Kaplan [6,10] of the nature of the activity of catalase fibers. We can assume that the activity of catalase fibers depends not on the true activity of catalase in the fibers, but of the native molecules adsorbed on them. However, since the fibers are structural formations, which may be distinct from the monolayer in their properties, this assumption must be tested. The solution of this question is offered in the next part of this study.

Fibers were obtained from the catalase monolayer by compressing its boundaries with barriers for 15 min after placing the protein on the surface. Investigation of the activity of the catalase fibers obtained on an underlying phosphate buffer, pH 6.0, showed that fibers taken from the cuvette surface and transferred to a reaction mixture (0.01 N H_2O_2 and phosphate buffer, pH 6.8) had some activity. However, this activity gradually

disappeared after numerous washings of the fiber in phosphate buffer, pH 6.8. Washing was carried out on a watch glass. If the activity of the unwashed fiber was 160 mmoles H_2O_2 in the sample, then after five washings it was 63 mmoles H_2O_2, after 10, 20 mmoles H_2O_2, and after 12, 7 mmoles H_2O_2 in the sample.

The disappearance of the fiber activity depends on desorption of native molecules of catalase from its globules, which is confirmed by the detection of this activity in the wash water.

As in the case of the monolayer, the activity of the fibers depends on composition and pH of the underlying layer (Fig. 3), which is connected with the amount of adsorption on the monolayer. As Fig. 3 shows, the fibers with the lowest activity are those obtained on an underlying layer of phosphate buffer, pH 6.8, in which the solubility of catalase is higher than in the buffer, pH 6.0. Fibers obtained on an underlying layer of 40% solution of $(NH_4)_2SO_4$ are characterized by the greatest activity, since because of the insolubility of the catalase in this liquid there are good conditions for adsorption. Then the activity of the unwashed fiber obtained on an underlying layer of 40% $(NH_4)_2SO_4$ is 1/6 the activity of the native catalase.

To show the share of adsorbed molecules of catalase in the residual activity of the fibers we used the following method: under a monolayer of serum albumin we placed a solution of catalase in an amount usually introduced on the surface when obtaining a monolayer of catalase. After the catalase had been carefully mixed in the underlying layer for 15 min, we took off the fiber of serum albumin and measured its "catalytic activity". Naturally, if the serum albumin fiber showed catalytic activity, this could be ascribed only to adsorbed molecules of native catalase on the monolayer. The activity should be higher, the greater the adsorption. As underlying solution we used a phosphate buffer solution with pH 6.0 and 6.8. The amount of decomposed H_2O_2 for the serum albumin fibers obtained at pH 6.0 was 175 mmoles H_2O_2, and at pH 6.8, 27 mmoles H_2O_2. The amount of H_2O_2 decomposed by catalase fibers obtained from a catalase monolayer with the same area as the monolayer of serum albumin at pH 6.0 was 160 mmoles H_2O_2 and at pH 6.8, 36 mmoles H_2O_2.

These results permit us to consider that the activity of catalase fibers depends entirely on adsorbed native molecules, that is, the catalase fibers themselves gave no activity.

Action of Shaking on the Enzyme Activity of Catalase Solutions

The effects which occur with shaking are basically due to surface denaturation analogous to that which occurs when a monolayer of protein is formed.

The experiments with shaking were carried out as follows: 0.0005% catalase solution in phosphate buffers with pH 6.0 and 6.8 were shaken on a shaking machine; after a determined time interval a sample was taken for determination of the activity. The results are given in Fig. 4. Inactivation of the catalase solution at pH 6.0 was almost instantaneous. After only 5 min shaking, the activity was 23% of the original value, and after 30 min, 12%. When the catalase was shaken in solutions with pH 6.8, the fall in activity was slight. After 3 hours of continuous shaking, the activity of the solution fell only 30%, which was evidently due to repulsion of the catalase molecules from the boundaries of the separate phases, since at this pH there is a charge. In solutions at pH 6.0 the charge on the molecule is slight, since this pH value is close to the isoelectric point of catalase (5.75).

The experiments with shaking once again demonstrate that spreading of the catalase molecules on surfaces is accompanied by loss of specific activity.

DISCUSSION

Our experiments lead to the following conclusions: spreading of catalase molecules at water-air interfaces is accompanied by complete loss of enzymatic activity, and the slight catalase activity of the monolayer and fibers is due to adsorption of native molecules. Hence, the conclusions of Kaplan on the retention of catalase activity by monolayers is erroneous, and the idea of Cheesman and Davies [17] that Kaplan was dealing with activity not of the monolayer itself, but of the native catalase molecule adsorbed on it, is actually true.

Thus, the fine structure of the protein component of catalase plays a great part in the catalytic function of this enzyme. The results agree with the data of the Japanese investigators [18] who showed that changes in the structure of the protein component under the action of various substances were accompanied by a fall in catalase activity. Since up to now the role of the protein component of the enzyme has not been sufficiently

studied, we are unable to decide accurately the nature of the structural changes which lead to loss of enzyme activity. The hypothesis suggested by Dounce and Fisher [19] to explain the high catalytic activity of catalase connects its properties with the presence of definite substrate-binding groups in the protein molecule near the prosthetic group. If this is so, then any destruction of this geometry will result in considerable decrease in the activity of the enzyme. It evidently follows that the loss of specific activity results from loss of the native configuration of the molecule when the monolayer is formed. Compounds like insulin, for which complete activity is retained in the monolayer [11] are the exception. The authors relate this property of insulin to the structural characteristics of its molecule.

Our results show that very great care must be exercised in relating the data of physicochemical investigations of monolayers to the interpretation of phenomena which occur in the living cell, since in monolayers an enzyme loses its activity completely and so the biological properties of the enzyme in the monolayer are very different from the biological properties of this enzyme in the living cell. In the living cell we must apparently speak of the adsorption of proteins on the cell structure accompanied more by distortion of the surfaces of the protein molecules than by their spreading.

SUMMARY

We have worked out a method for determining the enzyme activity of a monolayer of catalase directly in a cuvette, a method which permits us to deal with the entire unchanged monolayer of the enzyme with a large area.

Measurement of the enzyme activity of the catalase monolayer and fibers obtained by compressing the monolayer shows that spreading the native catalase molecule at an air-water interface is accompanied by its loss of specific enzyme activity.

The slight activity observed in the catalase monolayers and fibers depends on native catalase molecules adsorbed on the surface of the monolayer. This activity, that is, the amount of native molecules adsorbed on the monolayer, depends on the experimental conditions: pH and composition of the underlying liquid. The adsorption is higher at pH near the isoelectric point of the protein and in concentrated salt solutions (40% $(NH_4)_2SO_4$).

Inactivation of the catalase, observed with mechanical shaking of the enzyme solution, confirms the fact that spreading of the protein on the surface is accompanied by loss of specific activity, since the basis of the process which occurs with shaking is a surface denaturation.

The conclusion as to the retention of enzyme activity in catalase fibers which Kaplan [6,10] reached on the basis of his work is erroneous and is explained by insufficient removal of adsorbed native catalase molecules from the monolayer.

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THE PROBLEM OF CONTRACTILE PROTEINS OF SMOOTH MUSCLE

V. V. Oppel' and T. P. Serebrenikova

I. M. Sechenov Institute of Evolutionary Physiology, Academy of Sciences, USSR, Leningrad

When the proteins of a salt extract of smooth and cross-striated muscle are fractionated by salting out with ammonium sulfate, we find on the salting-out curve [1] a series of peaks [2] which correspond to the zones of salting-out of: actomyosin, myosin, n-meromyosin, tropomyosin, and nucleotropomyosin. Although the identification of proteins on the basis of one common property, in this case salting-out behavior, is impossible, all the same the presence of identical peaks makes probable the existence in smooth and somatic muscle of proteins which are very similar in structure, and, perhaps, in functional properties.

Besides these peaks, there are also regularly two peaks on the salting-out curve of smooth muscle which are not found on the curves of extracts of cross-striated muscle. One of these peaks corresponds to the zone of salting-out of free actin. It has been found in studying the contracile protein of the uterus [2] and esophagus [3] and is precipitated from a salt extract of the corresponding muscle by 10-fold dilution with water. The other peak has not been described before. It is very difficult to identify it with any known muscle protein.

In the present work we have set ourselves the problem of studying in more detail some of the proteins of smooth muscle.

METHODS

Stomach and thigh muscles of dogs, taken directly after the animal was killed, were minced with scissors with ice cooling. The minced muscle was extracted for a day, with shaking, in a refrigerator with a solution of 0.5 M KCl + 0.01 M KH_2PO_4 + 0.02 M Na_2HPO_4, pH 7.0-7.1 (ratio of solvent to minced muscle varied from 3:1 to 10:1), and then homogenized. The protein which dissolved was separated from the insoluble residue by passing through a linen cloth. The resulting muscle extract was further treated or was studied as such. The extract was kept in the cold.

The complex "contractile protein" was precipitated by 10-fold dilution of the extract with water. In separate experiments the extract was diluted four-five times. The precipitated protein was redissolved in 0.5 M KCl + phosphate buffer, and in some experiments was repeatedly reprecipitated, sometimes up to four times. The resulting protein, dissolved in buffered KCl, was kept at a temperature of -1 to 3°.

Protein was salted out from part of the extract by adding a saturated solution of $(NH_4)_2SO_4$ with pH 7 at room temperature to obtain a 20% saturated salt solution. After 15-20 min the precipitate was separated by centrifuging and at once dissolved in a small volume of 0.01 N NaOH. This solution was dialyzed in a collodion bag to full disappearance of ammonia in the liquid. In a number of experiments, ATP and ascorbic acid were added to the dialyzed liquid (at 40 μg per ml).

Myosin and actin were obtained from rabbit muscle. Actin was separated by the process of Straub. Separation of myosin was carried out by the method of Hasselbach and Schneider [4] using a solution of 0.01 M pyrophosphate which causes dissociation of actomyosin. This method permits getting active myosin with a large yield of the protein.

TABLE 1

Viscosity Number of Solutions of the Contracile Protein of Cross-striated and Smooth Muscle of Dogs. Concentration of protein in g per 100 ml

Cross-striated muscle		Smooth muscle			
precipitation by dilution with water (1:10)		precipitation by dilution with water (1:10)		precipitation by $(NH_4)_2SO_4$, 20% saturated	
Z_η	protein concentration	Z_η	protein concentration	Z_η	protein concentration
0.34	0.31	0.31	0.25	0.19	0.38
0.33	0.34	0.28	0.30	0.11	0.42
0.38	0.23	0.30	0.30	0.09	0.43
0.39	0.17	0.29	0.22	0.13	0.37
0.31	0.23	0.38(!)	0.27	0.14	0.36
0.38	0.22	0.22	0.22	0.13	0.47
0.41	0.35	0.28	0.33	0.09*	0.23
—	—	0.28	0.39	0.14*	0.11
—	—	—	—	0.17*	0.16
Average 0.36	0.26	0.30(0.28)	0.28	0.13	0.32

*Protected by ATP and ascorbic acid at the time of dialysis.

The protein content was calculated by nitrogen (N x 6.11). Fractional salting-out was carried out with a saturated solution of $(NH_4)_2SO_4$ with pH 7 at room temperature. The precipitate settled out and the supernatant liquid which contained unsalted out protein, was studied sprectrophotometrically at 278 $m\mu$ on an SF-4 spectrophotometer and the dynamics of the change (decrease) were followed by light adsorption with change in concentration of $(NH_4)_2SO_4$ ($-\Delta S/\Delta C$) depending on concentration of the salt (C).

The viscosity of the protein solutions was determined with an Ostwald viscosimeter; we used a buffered solution of 0.5 M KCl, pH 7.0-7.1. For dilution we used the same KCl solution. Calculation of the viscosity was carried out for definite protein concentrations which were determined by nitrogen. We calculated the values of relative viscosity (η_r), specific viscosity (η_{sp}) and the "viscosity number" (Z_η). The latter value was calculated by the formula $Z_\eta = 2.3 \log \eta_r / C$, where C is the concentration of the protein in g/liter [5].

In some experiments we studied the change in viscosity after adding to the protein solution in the viscosimeter the sodium salt of adenosine triphosphate (ATP).

RESULTS

In a number of parallel experiments we determined the "viscosity number" of the contractile protein of smooth muscle and actomyosin of cross-striated muscle. The results of the determinations are given in Table 1.

The value of the "viscosity number" of dog actomyosin agrees with the value given in the literature for rabbit actomyosin [5,6] and is somewhat less than for carp actomyosin [6].

The contractile protein of smooth muscle was precipitated in the same way as the actomyosin of cross-striated muscle, by tenfold dilution of the salt extract with water. The Japanese authors [3] proceeded in the same way in their study of the contractile protein of pig esophagus.

The average value of the "viscosity number" of smooth-muscle contractile protein (Table 1) is sufficiently close to that of the actomyosin of the dog cross-striated muscle (0.30 and 0.36). However, in the great majority of experiments, the value of Z_η for smooth muscle is considerably lower than for cross-striated muscle. The average value is strongly affected by the result of one experiment which is marked in the table, and which is excluded from the calculation of the average value given in parentheses.

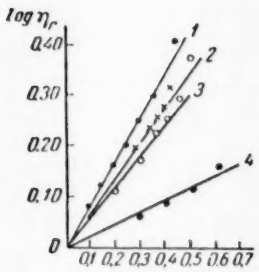

Fig. 1

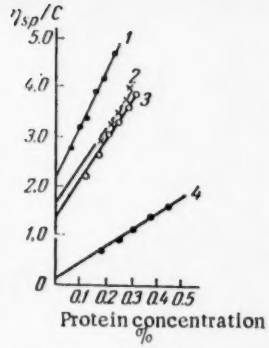

Fig. 2

Fig. 1. Change in log η_r (average values) when the protein solution is diluted. 1) actomyosin of cross-striated muscle; 2) smooth-muscle protein which remains in solution after salting out by 20% saturated $(NH_4)_2SO_4$; 3) smooth-muscle protein precipitated by dilution of the muscle extract with water; 4) actin of smooth-muscle salted-out by 20% saturated $(NH_4)_2SO_4$ solution.

Fig. 2. Change in η_{sp}/C (average values) when the protein solution is diluted. 1) actomyosin of cross-striated muscle; 2) smooth-muscle protein which remains in solution after salting out with 20% saturated $(NH_4)_2SO_4$; 3) smooth-muscle protein precipitated by dilution of the muscle extract with water; 4) actin of smooth-muscle, salted-out with 20% saturated $(NH_4)_2SO_4$.

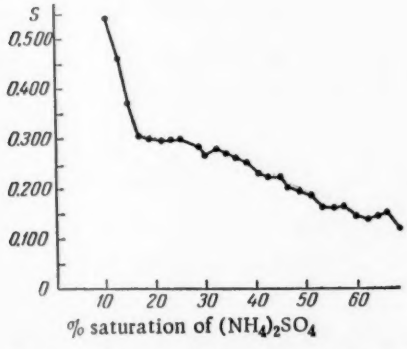

Fig. 3. Change in value of extinction of solutions of smooth-muscle protein at 278 $m\mu$ with increasing $(NH_4)_2SO_4$ concentration.

The comparative study of actomyosin and the smooth-muscle protein by the method of measuring change in viscosity when the solutions are diluted shows that the scattering of the figures in the experiments with smooth-muscle protein is much greater (two or more times) than in the experiments with the actomyosin of cross-striated muscle. This indicates the great inhomogeneity of the protein material in the first case. The change in log η_r with change of protein concentration in the solution (Fig. 1) indicates that actomyosin and the protein extracted from smooth muscle have a relationship of this index at small concentrations of the protein solutions which is characterized by the law given by Arrhenius and used successfully by Weber [7] in studying the proteins of cross-striated muscle. However, the numerical value of the constant in the formulas which described the behavior of actomyosin and the smooth-muscle protein is different (Fig. 1); in the case of the smooth-muscle protein, the angle formed by the curve (in the area of linear relationship) and the abscissa is much less. Also, the upper concentration of protein at which the viscosity of the solution begins to give a marked variation from a linear relationship is lower in an actomyosin solution than in a solution of the protein from smooth muscle, it is 0.35-0.40% in the first case and 0.45-0.50% in the second.

Exactly the same difference in steepness of rise of the curves is found in the graph of the relation of specific viscosity to total protein concentration (Fig. 2). We can assume from this that the asymmetry of the two contractile proteins is different (more for the actomyosin, less for the protein from smooth-muscle).

This raises the question of the composition of this contractile protein of smooth muscle. For an answer to this question we ran a series of experiments on fractional salting-out of the smooth-muscle protein which was precipitated from the extract by ten-fold dilution with water. In all, we used 29 different dilutions of saturated $(NH_4)_2SO_4$ in the interval from 10 to 70% of saturation. The results of these experiments indicated the presence of many peaks for proteins which were salted out, corresponding to peaks described in the direct salting-out of salt extracts of smooth-muscle. It is interesting that the peak for actin occurs particularly in the salting-out diagram.

We also carried out fractional salting-out of proteins reprecipitated three or four times by water. In these experiments, as in the preceding ones, there were numerous peaks on the salting-out diagram, and the actin peak was predominant in the precipitate.

Perhaps precipitation by dilution, even when repeated several times, is not suitable for purification of smooth-muscle contractile protein from the other proteins of the mixture. However, the possibility is not excluded that the smooth-muscle contractile protein is itself a protein complex which dissociates into its compo-

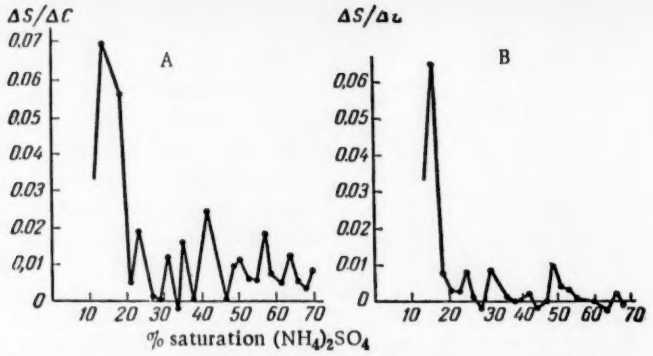

Fig. 4. Salting-out diagrams of smooth-muscle actin precipitated by 20% saturated $(NH_4)_2SO_4$. A) actin salted out once; B) actin salted out twice.

TABLE 2

Change in Viscosity when Rabbit Myosin is Mixed with Smooth-Muscle Actin from Dog Stomach. Smooth-muscle actin during dialysis was protected by ascorbic acid and ATP (neutralized) added at calculated values of 40 μg per ml of solution

Smooth muscle			Cross-striated muscle		
myosin	actin	myosin + actin	myosin	actin	myosin + actin
Protected during dialysis					
2.5(1)	0.27	5.37	1.04(1)	0.05	3.62
1.24(2)	0.38	1.62	0.52(2)	0.14	0.66
1.87(1)	0.19	4.34	0.88(1)	0.07	3.17
1.00(2)	0.18	1.18	0.52(2)	0.15	0.67
2.05(1)	0.32	3.22	1.58(1)	0 08	4.08
0.88(2)	0.26	1.14	0.62(2)	0 15	0.77
			0.60(1)	0.17	1.38
Unprotected during dialysis			0.34(2)	0.11	0.45
1.30(1)	0.35	1.70	0.65(1)	0.02	1.60
0.68(2)	0.20	0.88	0.49(2)	0.09	0.58
1.03(1)	0.20	1.11			
0.60(2)	0.15	0.81			
0.57(1)	0.22	0.88			
0.49(2)	0.15	0.64			

Note: (1) specific viscosity; (2) nitrogen content in mg/ml

nents only when it is salted-out with ammonium sulfate.

In distinction to Tonamura and Sasaki [3], we always observed in the smooth-muscle protein complex reprecipitated repeatedly by water a peak for tropomyosin which was salted-out in the zone of 50 to 60% saturation of $(NH_4)_2SO_4$. This observation agrees with the data of Snellman and Tenow [1] which were obtained in an investigation of the protein of uterus muscle.

We also tried salting-out salt extracts with ammonium sulfate for the separation of smooth-muscle protein. The supernatant liquid was carefully dialyzed against 0.5 M KCl until the reaction for ammonia disappeared. A study of the viscosity of the resulting solutions (Figs. 1,2,5) showed that the most viscous smooth-muscle component was not salted out with 20% saturated ammonium sulfate. The viscosity curve during dilution of solutions of the proteins which remained in solution after salting-out differed little (the viscosity was somewhat higher)

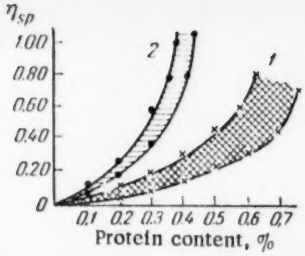

Fig. 5. Limits of variation of viscosity in smooth-muscle protein solutions with dilution. 1) actin salted out with 20% saturated $(NH_4)_2SO_4$; 2) protein which remains in solution after salting-out actin.

TABLE 3

Size of the Actin Peak ($-\Delta S/\Delta C$) in Salt Extracts of Smooth Muscle and Cross-striated Muscle of Dogs, with Different Degrees of Grinding. $(NH_4)_2SO_4$ concentration, 12-18% saturated. Nitrogen content in mg/ml

Experimental conditions	Smooth muscle		Cross-striated muscle	
	nitrogen content	$-\frac{\Delta S}{\Delta C}$	nitrogen content	$-\frac{\Delta S}{\Delta C}$
Minced with scissors	1.57	0.065	1.56	none
	1.15	0.042	1.06	"
	1.24	0.210	1.42	"
Minced in a homogenizer	1.49	0.049	1.26	0.023
	1.52	0.112	1.50	0.037
	1.78	0.168	1.76	0.028

from the curves which were obtained with the solutions of smooth-muscle proteins precipitated by water. Evidently the protein (or proteins) which remained in solution were salted-out at $(NH_4)_2SO_4$ concentrations close to those at which actomyosin and myosin of somatic muscle are salted-out. This is also indicated by the presence of the corresponding peaks (actomyosin, myosin) on the curve of fractional salting-out of the salt extracts from smooth-muscle [2].

According to the literature data [1-3], which are also confirmed by our work, there is always a peak on the salting-out curve for smooth-muscle proteins which falls at 10-20% saturation of $(NH_4)_2SO_4$. This peak is usually absent on the salting-out curves of cross-striated muscle. It corresponds to free actin in its position in the zone of 10-20% $(NH_4)_2SO_4$ saturation. We have studied this protein. The protein, salted out and separated by centrifuging, was dissolved in weak alkali, placed in a collodion bag, and dialyzed against 0.5 M KCl until the reaction for ammonia disappeared. Such a protein solution, strongly opalescent, was then studied further.

Fractional salting-out of the solution showed that it still contained a rather large amount of protein admixtures, and in 3 or 4 experiments we observed on the salting-out curve the same peaks as on the salting-out curves of the muscle extract. True, the size of these peaks, except for the primary and secondary ones (designated A and B in previous work [2]), was considerably less than in the experiments with extracts.

The experiments with repeated salting-out of the studied protein solution by 20% saturated $(NH_4)_2SO_4$ (Fig. 4) showed that in this way we could purify the protein sufficiently from admixtures of other proteins. In the study of the viscosity of these protein solutions we showed that the "viscosity number" in them (Z_η) was small and considerably lower than in actomyosin of cross-striated muscle on the contractile complex of smooth-muscle protein precipitated by tenfold dilution with water (Table 1). It approached the low value of Z_η of actin [5], and sometimes was even lower than this. Study of the change in viscosity of solutions of this protein during dilution showed still more scattering than in the experiments with smooth-muscle protein separated from the salt extract by dilution with water (Fig. 5).

Correspondingly, the curves which show relation of log η_r and ratio η_{sp}/C to concentration of protein in solution (Figs. 1 and 2) fall somewhat lower than curves for actomyosin solution and solutions of the proteins of the smooth muscle complex precipitated by dilution with water. This indicates the considerably lower asymmetry of the protein molecule salted out by 20% saturated $(NH_4)_2SO_4$ from the salt extract of smooth muscle than of the particles of actomyosin and contractile protein complex of smooth muscle. The scattering of the different curves evidently indicates a different degree of denaturation of the protein.

The study of the viscosity of this protein when ATP is added shows its insensitivity to adenosine triphosphate, a fact which permits us to consider that the smooth-muscle protein salted out by 20% saturated $(NH_4)_2SO_4$ can hardly, in itself, be the contractile protein complex of this muscle.

The fact that in the solution of salted out smooth-muscle protein the viscosity is small, and there is no

particular change when ATP is added leads to the conclusion that we are dealing with salted out actin, depolymerized and partly or completely denatured during the dialysis. As is known, with a long dialysis of actin without protection of its "active centers", it undergoes depolymerization and denaturation [8].

To settle this question we ran several sets of experiments on measuring the change in viscosity of the following protein solutions in combination: 1) myosin + rabbit actin; 2) myosin + rabbit actin salted out with $(NH_4)_2SO_4$, 20% saturated, followed by long dialysis against 0.5 M KCl solution; 3) rabbit myosin + smooth-muscle protein salted out with the above concentration of $(NH_4)_2SO_4$ and dialyzed without protection of the "active groups"; 4) rabbit myosin + salted out smooth-muscle protein dialyzed with addition of ATP and ascorbic acid. Some examples of these experiments are given in Table 2.

These data indicate that smooth-muscle protein salted out by 20% saturated $(NH_4)_2SO_4$ is really actin. This is particularly shown by its positive reaction with rabbit myosin in the experiments with protection of the "active groups" during dialysis. Its inactivation during dialysis which accompanies the salting out, when the "active centers" of the protein remain unprotected shows a similarity with the development of corresponding processes during the dialysis of the actin from cross-striated rabbit muscle [8].

The relatively high viscosity of actin solutions from smooth muscle deserves attention. The question of whether this is due to the presence of other protein admixtures (actin was salted out only once in these experiments) requires further study. Experiments with salted out actin from cross-striated rabbit muscle after long dialysis carried out without protection with ATP and ascorbic acid were unsatisfactory because of the strong denaturation of the actin and the almost complete loss of its solubility.

DISCUSSION

At the present time a number of facts have accumulated in the literature which are interpreted by many authors as indirect confirmation of the presence in smooth muscle of free actin; these include: the appearance on the electrophoretogram of a peak which resembles the peak of free actin [9]; the detection in smooth muscle of a protein salted out by the same concentration of ammonium sulfate (20% saturated) as actin [2,3]; the ability of a solution of myosin A from cross-striated muscle to extract from smooth muscle an "actomyosin" whose fibers contract with ATP [10]. The present work finally establishes this by the separation of actin by salting-out and obtaining from its mixture with myosin an actomyosin which has a very high viscosity.

The experiments with $(NH_4)_2SO_4$ salting-out indicate the presence in the salt extract from smooth muscle of free actin. At the same time, the results of the experiments with repeated precipitation of the smooth-muscle contractile complex by ten times its dilution with water force us to use care in referring to completely "free" actin. Actually, 3-4 reprecipitations of the smooth-muscle protein by water do not markedly decrease the content of actin in the reprecipitated protein, while it is known that depolymerized actin is a water-soluble protein. We can believe that there is in the protoplasm of the smooth-muscle cell some very labile bond between the different proteins, which is partly maintained in the salt extract (0.5 M KCl). This bond is broken in salting-out, but is retained when the protein is precipitated by decreasing the ionic strength of the solution.

It is known from the literature that the actin of cross-striated muscle can be removed from the muscle by treatment with acetone only when the mincing of the muscle is not very intense. The use of careful homogenization of the muscle results in transfer of the actin to the aqueous salt solution, which eliminates the need for preliminary acetone treatment [4]. In this connection, we repeated our experiments with smooth muscle and cross-striated muscle of dogs with strict control of the degree of mincing. Some of these experiments are shown in Table 3.

As can be seen, careful grinding of the cross-striated muscle in the homogenizer accompanied by destruction of the cell structures actually results in the appearance of actin in the salt extract of the muscle, while none is present in the extract from the roughly minced muscle. However, in the homogenate, the amount of actin is somewhat less than in the experiments with homogenized smooth muscle, and does not even reach the level of actin in the extract of smooth muscle submitted to rough mincing. This shows the characteristic state of actin in smooth muscle: its "freer" state, perhaps, and its greater content in the muscle.

Actin itself does not assure muscular contraction. For this to occur it should obviously always form a complex compound with a protein of the type of myosin. The viscosity of actin isolated from salt extracts of smooth muscle is not particularly changed when ATP is added. This also indicates indirectly its inability to contract.

The idea of Huxley [11,12], based on electron microscope data and on the shortening of "fine" actin fibers of cross-striated muscle during its contraction still requires a chemical interpretation and meanwhile does not change our ideas of the mechanically active actomyosin complex.

However, the presence in the contractile protein complex of smooth muscle, three times reprecipitated by water, of a definite actin peak, considerably larger than the peak of the remaining proteins (shown by the relatively greater light absorption of actin at 278 $m\mu$ compared to the other proteins of the muscle [14]), suggests strongly the possible presence of some other ratio of components in the contractile protein complex of smooth-muscle than that which occurs in the actomyosin of somatic muscle.

The present work does not settle the question of the nature of the contractile complex in smooth-muscle. The protein precipitated from salt extracts of smooth-muscle by dilution of this extract with 10 times the amount of water and which has been assumed by other authors [1,3] to be the contractile protein of smooth-muscle by analogy with actomyosin, is quite unhomogeneous as shown by the viscosity of its solution and by the picture which is obtained in fractional salting-out. As mentioned above, actin is always present in it. The viscosity of its solutions as a rule is somewhat lower than the viscosity of actomyosin solutions.

The extreme scattering of the data when a series of studies is made of changes in the viscosity of solutions of this protein with dilution can be the result of the lack of homogeneity of the protein composition in different experiments, as Ivanov and Kiseleva [10] have assumed for smooth-muscle extracts. Another possibility is that the free actin of this protein precipitate, denatured differently in different experiments, does not correspond to the control and its viscosity is affected differently. We should not forget that on the electrophoretogram of the smooth-muscle extract the peaks which correspond to the peaks of actomyosin and myosin in cross-striated muscle have a very wide base, which indicates their relative heterogeneity [11].

It would seem that the fractional salting-out has finally confirmed the presence of many components in the smooth-muscle "contractile protein" which is precipitated by water. Numerous peaks for different proteins have been noted here. However, the retention of these peaks after repeated precipitations by water, including the peaks for tropomyosin and actin (proteins which are easily soluble in solutions with low ionic strength) raises the question of the possible existence of weakly bound complexes of this protein, from which the separate components may be isolated by salting-out.

The work of Huxley [12,13] has shown a double character for the contractile fibers in muscle: thick and thin, of which the first belongs to myosin and is located in disk A, while the second, actin, is distributed through disks I and A; this seems to apply only to cross-striated muscle. An electron microscope picture for smooth muscle, obtained with carefully prepared sections, (thickness of the section, calculation of the angle formed by the plane of the section with the long axis of the fiber) has not been described in the literature as far as we know. If the theory of Huxley is correct in this respect, then the proteins of the contractile complex of cross-striated muscle, actin and myosin, are spatially isolated from each other in separate fibers connected only by protoplasmic bridges (the place of the active centers of ATP-ase) distributed at definite distances from each other; and does it not follow to consider a more diffuse distribution of the proteins of the contractile complex within the threads of the smooth-muscle contractile fibers? There is no inhomogeneity in the structure of muscle fibers in smooth muscle. Both functionally and in an evolutionary sense it is a less highly differentiated contracting tissue. Perhaps this lower differentiation is expressed in a more even arrangement in the contractile fiber and their contractile protein threads which make up the contractile complex. From this point of view we can understand the slowly developing "vermiform" contractile reaction of smooth-muscle; the diffuse arrangement of the actin and "myosin" in the threads of the smooth-muscle fiber fibrils should give a diffuse contracting reaction.

SUMMARY

Smooth-muscle differs from cross-striated muscle in showing actin in a salt extract of the tissue even when it is coarsely minced. The "contractile protein" of smooth-muscle, precipitated by diluting its salt extract with water, differs from the actomyosin of cross-striated muscle by its lower viscosity. The heterogeneous composition of this contractile protein raises the question of the complicated structure of the complex.

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A COMPOUND LIPID COMPLEX EXTRACTED FROM DIPHTHERIA MICROBES BY EXTRACTION WITH BENZENE

E. K. Alimova (Lubenets)

Department of Biochemistry, Rostov-on-Don Medical Institute

We have previously extracted from the free lipids of diphtheria microbes PW-8 a fraction A (scheme) [1], in whose composition we have detected a high molecular weight fatty acid and a carbohydrate which is liberated only by hydrolysis. On the basis of these results the fraction was considered to be a complex compound.

In this study we give data obtained as a result of chemical study of this complex.

Obtaining the Complex from the Benzene Extract

For isolation of the above-mentioned compound we used bacteria grown by the same method as when we isolated the free and bound lipids [1, 3-5]. They were filtered from the medium, washed on the filter with physiological saline, and dried at 37°. The dried microbe mass was defatted with benzene or ether in a Soxhlet type apparatus.

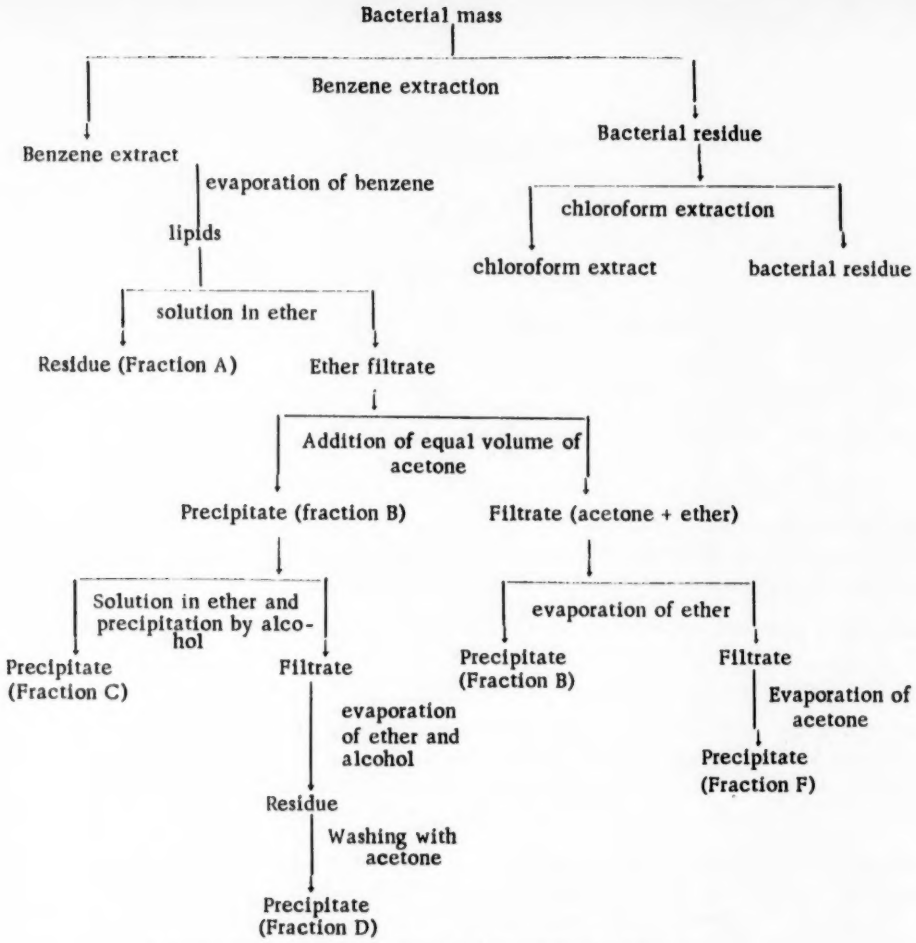
The solvent was removed from this extract in a stream of carbon dioxide. Repeated solution of the residue in ether precipitated delicate white flakes which were separated by filtration from the residual extract and carefully washed with ether. The resulting substance was not soluble in benzene, ether, alcohol, or chloroform. It was also insoluble in water, dilute acids, or alkalies. It had the form of dense white scales with melting point 65.5-77.5° and it reacted with reducing substances only after hydrolysis. The substance gave the Molisch reaction for carbohydrates and the Bial reaction for pentoses. The biuret reaction was positive only after partial hydrolysis (grinding with a concentrated sulfuric acid solution, 2:1, at room temperature). When the substance was given a long heating with 1% ninhydrin solution, it showed a blue color which was extracted by amyl alcohol, and evolved a free fatty acid.

In this substance we found: 9.8% ash, 0.32% calcium, 0.27% magnesium, 0.15% phosphorus, 1.0% total nitrogen according to Kjeldahl, and about 64% lipids. In order to study the composition of this substance, we saponified 3.81 g of it for 10 min with 0.5 N alcoholic KOH in the presence of 15 ml of benzene. Saponification was carried out for a day in the cold and for 6 hours on a boiling water bath. Then the alcohol and benzene were removed, and the insoluble portion was again saponified with 10 ml of normal alcoholic KOH for 4 hours on a boiling water bath. As a result of the saponification, fraction A was separated into substances soluble in a mixture of alcohol and benzene, amounting to 63.7% and substances insoluble in this mixture, amounting to 36.3%.

Substances Soluble in a Mixture of Alcohol and Benzene

In the composition of substances soluble in a mixture of alcohol and benzene we detected high molecular weight fatty acids which were separated into four fractions [1]. The properties of these are given in the table.

Qualitative analysis of each fraction was carried out by single-phase diffusion paper chromatography [2] and by this method we detected in the second and third fractions palmitic acid. In the fourth fraction, when the spot was fixed with bismuth we found two spots on the chromatogram impregnated with vaseline oil: one corres-



Scheme of Fractionation of Diphtheria Bacteria Lipids

Properties of the Fatty Acid from the Alcohol-Benzene Solution

Fraction No.	External form	Color	Molec. Wt. determined by neutralization no.	Melting point	Refraction index	Optical activity	Iodine no.
1	Solid, powdery mass	Colorless	567.6	70.4	1.439	Has none	0
2	»	»	320.2	57.5	1.4335	»	0
3	»	»	293.8	59.7	1.432	»	0
4	Solid, nonpowdery mass	Light yellow	321.7	49.1	1.441	»	30.6

ponded to palmitic, the other to hexadecenoic acid.

When we developed the chromatogram with a manganese solution, we detected only one spot, which corresponded to hexadecenoic acid. In chromatographic study of the first fraction we obtained on the chromatogram impregnated with vaseline oil a difficulty diffusing spot of fatty acids. When we used a chromatogram impregnated with paraffin we observed traces of myristic acid, somewhat more palmitic acid, C_{20-22} acids, and a high molecular weight acid which did not move from the point of deposition.

The high molecular weight fatty acids of the second, third, and fourth fractions determined by the neutralization number compared to palmitic acid determined by the chromatographic method, were certainly due to admixtures of the first fraction.

The presence of unsaturation, hexadecenoic acid in the fourth fraction, was also confirmed by the iodine number and the increased index of refraction compared to palmitic acid.

Substances Insoluble in the Mixture of Alcohol and Benzene

In the substances insoluble in a mixture of benzene and alcohol we found a polysaccharide which was purified by obtaining the acetone derivative with later regeneration of the carbohydrate [1]. The carbohydrate gave the Molisch reaction, and a weak Bial reaction.

By single-phase paper chromatography after hydrolysis of the polysaccharide, we detected one spot which corresponded to the spot of mannose. Since the pentose reaction of the substance was positive, but paper chromatography did not show a pentose after the above separation of the carbohydrate, we tried a new separation of the polysaccharide by saponification of the substance under investigation. For this purpose, 1 g of the substance was hydrolyzed with a 40% alcoholic solution of alkali on a boiling water bath for two hours. The alkali solution was poured off, and the residue which settled to the bottom of the flask was dissolved in water and precipitated by methyl alcohol. After hydrolysis of the precipitate with 1 N HCl for four hours, it was diluted with water and at once passed through a column of acid resin, then through a column of basic resin. The filtrate was concentrated in a vacuum. The polysaccharide hydrolyzate was studied by paper chromatography and showed the presence of only one spot which corresponded to mannose.

Since the substance being studied gave a reaction with ninhydrin and 1.0% nitrogen was found in it, we studied its content of amino acids. We did not succeed in extracting from this substance a protein either by extraction with 90% phenol or with picric acid. Then 0.7 g of the substance was hydrolyzed with 6 N HCl for 24 hours on a boiling water bath. The acid-soluble part of the hydrolyzate was separated, and after removal of the acid, was used for amino acid analysis. By the method of paper electrophoresis and single-phase ascending paper chromatography, we detected 12 amino acids: aspartic, glutamic, alanine, threonine, glycine, serine, two diamino acids, cysteine, phenylalanine, valine, and leucine.

From all these data it is evident that this is a complex compound of fatty acids of high molecular weight, polysaccharide, protein (polypeptide), and mineral substances. Only mannose was found in the composition of the polysaccharide. A similar type of polysaccharide, consisting only of mannose, has been found in our laboratory by Orlova [6] in the composition of whole cells of diphtheria microbes. This complex is distinguished by the absence of solubility in ordinary fat solvents, including those used in its isolation, and its stability towards acids and alkalies with heating. The loss of solubility in fat solvents can be explained by the loss of bonds with other lipids in the course of the treatment of the benzene extract (evaporation, drying, etc.). If there were no bonds between the different components of the extract, but it was only a complex mixture of one lipid with another, which assured their solubility, then this would be restored when the isolated components were mixed. However, in our experiments we did not restore the solubility of the complex by preparing different complex mixtures of the lipids isolated from the benzene extract. The large amount of mineral matter observed in the complex is especially important (about 10% ash), including, as it does, calcium and magnesium. These minerals could not enter the complex from the medium or from the physiological saline used to wash the bacteria. Special experiments showed that sodium chloride was not extracted by benzene which was used in extracting the bacterial mass. From the conditions of extraction and the other properties of the complex it is quite evident that the mineral substances are bound to the fatty acids and other components of the complex. Probably, the presence of inorganic ions with a definite charge binds a complex, particularly stable toward acid and alkali with heat. We can assume that the formation of a compound stable lipid-mineral complex is a characteristic mineral depot for the bacterial cell.

Since this study of such compounds, complex in character, therefore also in the nature of their bonds, is of definite biological interest, we consider that this complex should be the object of further study.

SUMMARY

The benzene extract obtained from diphtheria microbes PW-8, gave a fraction insoluble in benzene, ether,

alcohol, and chloroform. This fraction A is a complex compound composed of higher fatty acids (about 64%), mineral substances (9.8% ash), protein (polypeptides, 1.0% total nitrogen), and a polysaccharide which is composed of mannose.

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THE TRANSAMINATION OF GLUTAMIC ACID IN THE DEVELOPING CHICKEN EGG

K. A. Drel'

Department of Biochemistry, Lugansk Medical Institute, Lugansk

In the study of the effects of different physiological and pathological factors on the transamination of glutamic acid, Vyshepan [1] observed that the transamination process neither decreased nor increased, not only in the presence of various enzyme poisons, but also with very deep destruction of the tropic state and morphological structure of organs (alimentary muscular dystrophy, fatty degeneration of the liver, etc.). On the basis of his results, the author excluded the possibility of a special role of the transamination process in the contracting action of the muscle.

In explaining the absence or very slight activity of transamination in a number of organs with a highly specialized metabolism, as opposed to its considerable intensity in tissues with less specific chemistry, Braunshtein [2] also assumed that these followed more generalized biological rules in the relation between nitrogen metabolism and differentiated biological function; transamination was more widely distributed in living organisms than the ability for oxidative deamination and the synthesis of amino acids from NH_3 and keto acids.

In summary, the transamination process should play an important part in the formation of bird embryos from the proteins which are contained in the egg. The formation of different, highly specialized organs, their differentiation and development are possible with intensive transformations of the amino acids of the yolk and the white [4-8]. All the amino acids needed to form the embryo are contained in the yolk and white of the hen egg [3-7], but this does not exclude a change in their ratios as a result of processes of splitting and synthesis of amino acids in the developing egg. Perfectly definite data have been obtained [8-11] on the increase or decrease in the content of certain amino acids during embryonic development. For example, it has been shown that the amounts of tryptophan, arginine, and cystine decrease during development of the egg, and the amounts of histidine, lysine, and others increase. The content of dicarboxylic amino acids rises during the first half of incubation, and in the second half falls sharply. A considerable change has also been observed in the ratio of amino acids in the yolk, white and embryo at different stages in the development of the egg. The question of the change in the transamination reaction during development has been little studied.

Shmerling [12] studied transamination in the organs of the rabbit embryo. He showed that in different embryonic organs transamination increased differently. The author concluded that the increase in the transamination reaction in different organs was not regularly related to the rate of growth of these organs or the degree of their morphological differentiation. Shmerling also confirmed that the activity of transamination in the organs of the embryo was weaker than in the organs of the mature animal. The low activity of aminopherases was retained in the early postembryonic period. In later work [13], Shmerling showed that in the early stages of development when there is still no transamination in the embryo, it is already found in the placenta. Thus, the transamination process certainly takes part in the development of the embryo. In all stages studied, transamination was found in the rabbit placenta, while in the embryonic tissues it appeared comparatively late. In the rat placenta this reaction was completely absent, but the tissues of the rat embryo at all stages had the power of transamination. In the experiments on the developing egg it was established that the transamination enzyme system from the beginning of incubation was found in the yolk, while it was absent in the tissues of the embryo [13].

TABLE 1

Change in Quantity of Dicarboxylic Amino Acids and Alanine in the Reaction Mixture after Incubating the Yolk of Unfertilized Eggs with Glutamic and Pyruvic Acids

Day of incubation	No. of eggs studied	Dicarboxylic amino acids	Alanine
		micromoles/g of yolk	
Before incub.	5	−32.0	+60.8
5th day	5	−16.7	+26.2
10 »	5	−29.0	+70.0
15 »	6	−13.3	+30.0
20 »	6	−19.3	+19.1

TABLE 2

Change in Quantity of Dicarboxylic Amino Acids and Alanine in the Reaction Mixture after Incubating the White of Unfertilized Eggs with Glutamic and Pyruvic Acids

Day of incubation	No. of eggs studied	Dicarboxylic amino acids	Alanine
		micromoles/g of white	
Before incub.	5	−28.5	+32.4
5th day	4	−9.1	+10.6
10 »	7	−12.4	+10.6
15 »	8	−2.7	−9.7
20 »	10	−9.6	−32.4

TABLE 3

Change in Quantity of Dicarboxylic Amino Acids and Alanine in the Reaction Mixture after Incubating the Yolk of FertilizedEggs with Glutamic and Pyruvic Acids

Day of incubation	No. of eggs studied	Dicarboxylic amino acids	Alanine
		mmoles/g yolk	
Before incub. - 5th day	5	−42.0	+54.2
	6	−30.6	+57.6
10 »	7	−36.0	+63.0
15 »	6	−13.3	+15.4
20 »	4	−34.1	+37.4

TABLE 4

Change in Quantity of Dicarboxylic Amino Acids and Alanine in the Reaction Mixture after Incubating the White of Fertilized Eggs with Glutamic and Pyruvic Acids

Day of incub.	No. of eggs studied	Dicarboxylic amino acids	Alanine
Before incub.	5	−25.8	+23.4
5 th day	6	+ 1.0	+18
10 »	5	−16.0	+31
15 »	5	−17.8	+25,6

Some other results have been published by American authors [14]. These authors observed glutamic transaminase in the yolk sac already after 24 hours of incubation. Its activity increased up to the second half of the incubation period, after which the activity of the enzyme fell off. In the tissues of the embryo, on the other hand, the activity increased in parallel with the increased protein content in the embryo. The distribution of the transamination enzyme activity in different tissues of the chicken embryo was unequal and changed during the development.

The literature data on the study of transamination in the developing hens egg are limited to these two sources. Obviously, the above investigations are far from sufficient to give a definite idea of the occurrence and development of the transamination process in the developing hens egg, and its significance in the formation of the embryo.

In the present investigation we have attempted to study the activity of glutamic transaminase in the incubation of fertilized and unfertilized hens eggs.

METHODS

The study of fertilized and unfertilized hens eggs was carried out with White Leghorns. The transamination process was studied separately in the white and yolk in unfertilized eggs, and in the fertilized eggs, in the embryo, the yolk up to 20 days of incubation, and the white up to 15 days of incubation, that is, while it was possible to separate the white and the yolk.

The eggs were studied on each 5 days of incubation and also when freshly laid, before placing in the incubator. The activity of glutamic transaminase was studied in the egg tissues during incubation in solutions of

a mixture of glutamic acid and pyruvic acid. In each experiment the reaction mixture contained 200 μ moles of LD-glutamic and pyruvic acids, first neutralized to phenolphthalein with alkali. The presence of the transamination process was judged by a fall of glutamic and pyruvic acids and increase in the fraction of amino nitrogen of monocarboxylic amino acid (alanine) [15]. Incubation of the tissues was carried out at 37-38° for two hours. The samples were treated with monobromoacetic acid to prevent glycolysis.

Dicarboxylic amino acids were determined according to the method of Kretovich and Bundel' [16]; total amino acids, by the method of Folin. Pyruvic acid was determined colorimetrically with salicylic aldehyde [15]. The increase in alanine during transamination in the reaction mixture was determined by the difference in total amino nitrogen and nitrogen of the fraction of dicarboxylic amino acids. The loss in glutamic and pyruvic acids and the increase in alanine were calculated in μ moles per g of studied tissue.

RESULTS

The results of the investigation of transamination in unfertilized eggs at different periods of incubation are given in Tables 1 and 2. The data of Table 1 show the intensity of transamination in the yolk of unfertilized eggs.

These figures indicate the presence of the transamination process in the yolk of unfertilized eggs during 20 days incubation, and that the most intense occurrence of this process is on the tenth day of incubation.

In the transamination of glutamic acid, Braunshtein and Kritsman [17] always obtained an equivalent amount of alanine. The excess of alanine in our experiments can be explained by the not entirely specific method which we used for its determination.

In Table 2 we give the figures obtained in the analysis of the white of the unfertilized egg.

As in the yolk, transamination was observed in the whites of fresh, unincubated eggs. Here the decrease in dicarboxylic amino acid corresponds to the equivalent increase in alanine.

With 5-days incubation we found some decrease in amount of dicarboxylic amino acid and an equivalent increase in alanine. On the tenth day we also observed a decrease in amount of dicarboxylic amino acids in the reaction mixture, equivalent to the increase in alanine.

On the 15th and 20th days of incubation we observed a decrease in the amount of alanine and dicarboxylic acids, which permits us to consider the absence of transamination.

The results of the study of the transamination process in fertilized eggs are given in Tables 3-5. In the yolk of fertilized eggs, as in the yolks of unfertilized eggs, we found transamination of glutamic acid at all stages of development. As distinct from the yolks of unfertilized eggs, Table 3 shows that transamination occurs with great intensity in the yolks of fertilized eggs.

Table 4 gives results obtained by determining the transamination in the whites of fertilized eggs.

In this series of experiments, after 5-days of development of the egg, we could not show the presence of transamination between glutamic and pyruvic acids. At 10 and 15 days incubation we observed a fall in dicarboxylic acids and a rise in alanine in the whites of fertilized eggs.

Table 5 gives the figures for activity of the transamination enzyme in the embryo at all stages of incubation.

As the table shows, transamination is observed in the homogenate of chicken embryos during the period of their development.

DISCUSSION

According to our data, in the developing hen egg, glutamic transaminase is present in the tissues of the embryo, the yolk, and the white. We found the greatest activity of glutamic transaminase in the homogenate of the whole embryo. The following figures indicate the intensity of the transamination process in the embryo: of 200 μ moles of racemic glutamic acid the homogenate of a twenty-day-old embryo transaminated 47 μ moles, that is, almost 50% of the L-isomer, which, according to the calculations of Kritsman [18] is a high activity of transamination.

TABLE 5

Change in Quantity of Dicarboxylic Amino Acids and Alanine in the Reaction Mixture after Incubation of the Embryo Components with Glutamic and Pyruvic Acids

Day of incubation	No. of eggs studied	Dicarboxylic amino acids	Alanine
		mmoles/g	
5th day	7	—111.0	+242.0
10 »	8	—40.4	+66.0
15 »	6	—35.6	+52.0
20 »	7	—47.0	+79.7

Somewhat lower activity of glutamic transaminase was noted in the yolk of the developing egg. On the average it was 30-35% and only on the 15th day of development did it fall to 13%. In the white of fertilized eggs we observed still less activity of this enzyme; thus, on the 5th day activity was almost absent, and on the 10th and 15th days did not pass 16-17%. In distinction from the literature data, we observed a sufficiently high activity of glutamic transaminase in unfertilized eggs: 42% in the yolk and 25% in the white.

It is very likely that in the yolk and white of the developing egg there occurs by transamination a redistribution of the nitrogen corresponding to its use by the embryo at different stages of its development.

The presence of glutamic transaminase activity in the unfertilized egg indicates that this enzyme accumulates even in the formation of ova in the ovaries. Fertilization can only hasten or strengthen the already existing activity. This is confirmed by the data showing the same transaminase activity in fresh, unincubated eggs, whether fertilized or unfertilized.

However, in the yolks of unfertilized eggs, the glutamic transaminase activity is much less than in fertilized eggs, although the character of the change in activity remains the same, namely, that the lowest activity in the yolk of unfertilized eggs is found on the 15th day, as is also noted for the yolks of fertilized eggs. In the whites of unfertilized eggs we observed a very low activity, not surpassing 9-12%, in the first half of the incubation period only.

One of the factors which affects the change in transaminase activity may be a change in pH in the incubating egg. Kritsman [18] showed that this enzyme is active in the pH zone from 5.5 to 8.0; its optimum action occurs about 7.4, and at pH 9.0 it is inactivated.

During the development of the egg, as Shklyar [19] and Ermakov [20] showed, the pH of the yolk regularly rises, and that of the white falls. In the yolk the reaction medium does not exceed the limits of pH values in which transaminase is active. In the whites of fertilized eggs during the first four days of development, the pH is above 9.0 where glutamic transaminase is inactive. In just this period of development of the egg we find negligible transaminase activity of the white.

In our previous work [21] where we studied the content of free dicarboxylic amino acids and total amino acid nitrogen in unfertilized eggs, we denied the possibility of synthesis or hydrolytic splitting of the proteins of yolk and white. We came to this conclusion on the basis of the constancy of amino acid composition of the unfertilized egg during 20-days incubation. This conclusion seems to contradict the data of the present work on the presence of transamination processes, that is, the transformation of proteins in unfertilized eggs. However, such a contradiction can be easily explained. We consider that transamination is undergone chiefly by free amino acids, which are always contained in the whites and yolks in considerable amounts. Also, Braunshtein [12] suggested the possibility of a transfer of amino nitrogen by transamination from longer carbon chains of the higher amino acids to shorter ones, as a result of which part of the carbon of the protein is oxidized with simultaneous retention of the amino nitrogen. Further, in the previous work, we found a small change in the content of free dicarboxylic amino acids which we ascribed to an inaccurate method. In the light of the new data on transaminase activity in unfertilized eggs, another explanation of the variation in amino acid nitrogen is not excluded, namely, the transformation of the dicarboxylic amino acids into monocarboxylic acids which occurs, though weakly, in the yolks of unfertilized eggs.

SUMMARY

In homogenates of whole chicken embryos we have found a high activity of glutamic transaminase at all stages of development.

In the yolks of fertilized eggs we have found a lower activity of this transamination enzyme, which changes during incubation. In the whites of fertilized eggs a high activity is found only before incubation; on the 5th day it is completely absent, and on the 10th and 15th days it is very low.

Transamination of glutamic acid is observed also in the yolks and whites of unfertilized eggs. In unfertilized eggs glutamic transaminase activity is lower than in fertilized eggs, but the character of the change of enzyme activity during incubation is identical for fertilized and unfertilized eggs.

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ON THE EFFECT OF THE AMINO ACID SIDE CHAIN AND OF THE ACYL RADICAL ON THE ENYZMATIC SYNTHESIS OF L-ACYLAMINO ACIDS BY ACYLASE I.

B. N. Orekhovich, L. A. Shchukina, Chi Cheng-Wu and V. B. Spirichev

Institute of Biological and Medical Chemistry, USSR Academy of Medical Sciences, Moscow

It was shown in previous communications [1, 2] that kidney acylase I could catalyse not only the hydrolysis [3, 4], but also the synthesis, of N-acetyl-L-alanine.

The present work was undertaken in order to determine the degree to which the acylation of other amino acids was catalyzed by this enzyme, and also to establish the effect on the reaction of the structure of the side chain of the acylated acid. Moreover, since Greenstein et al. [5-7] have demonstrated that acylase I catalyzed the hydrolysis of acyl-α-amino acids other than the N-acetyl derivatives, it was of interest to establish the degree of specificity of this enzyme for the acylating group in the synthesis of the acyl derivatives, and, in the case of carboxylic acids, the effect of the aliphatic side chain.

The problem was approached in two experimental series. In the first experiment acylase I was incubated with sodium acetate throughout, but in the presence of one of 18 different amino acids. Where acyl amino acid synthesis took place, the initial reaction velocity, per cent synthesis at equilibrium and the equilibrium constants were calculated.

In the second experimental series, the catalytic effect of the acylase I in the acylation of L-leucine was tested with a variety of carboxylic acids. Here also the per cent synthesis of acyl-L-leucine and the equilibrium constant were calculated.

EXPERIMENTAL

The conditions for the acylation reaction were as described in a previous communication [2].

In the experiments with the various amino acids the initial concentration of the Dl-amino acids was 0.05 M, with the exception of DL-leucine (0.0375 M) and DL-tryptophane (0.0275 M), the concentration of which was limited by their solubility. The concentration of sodium acetate was 2.5 M throughout.

In the experimental series involving the acylation of L-leucine by the various carboxylic acids, the former was present at a concentration 0.0375 M, while the acylating acids were present at a concentration 2.5 M. In this experimental series, unlike in the experiments mentioned above, the acylating acids were not added as their sodium salts, but as the free acids, which were then neutralized by the addition of 6 N sodium hydroxide. The solutions were then accurately adjusted to pH 7.5 by the addition of a few drops of either dilute sodium hydroxide, or of the carboxylic acid used. No changes in pH were observed during the incubation period. Acylase I was added to the incubation mixtures as a dry, purified preparation [8, 9].

The degree of acylation was assayed by the disappearance of amino nitrogen as determined by Van Slyke.

TABLE 1

The Enzymatic Acetylation of Various L-Amino Acids

Amino acid	Acetyl-L-amino acid synthesized at equilibrium, %	Equilibrium constant	Initial reaction velocity, μM /h/ mg acylase N
Tryptophane	13	0.06	<5
Glutamic acid	15	0.07	14
Alanine	21	0.11	27
Norleucine	25	0.13	35
Norvaline	27	0.15	64
Aminobutyric acid	28	0.16	68
Ornithine	20–30	0.10–0.17	<5
Valine	34	0.21	21
Histidine	34	0.21	10
Leucine	35	0.22	90
Serine	37	0.24	28
Asparagine	39	0.26	10
Phenylalanine	40	0.27	17
Threonine	40	0.27	28
Lysine	30–40	0.17–0.27	<5
Glycine	42	0.29	27
Methionine	48	0.37	250
Aspartic acid	0	0	0

Note: The figures in the Table represent mean values of 3-4 determinations, where individual variations did not exceed 5%; exceptions from this were ornithine and lysine in which, because of the high nitrogen-group content, the accuracy of determination was low, so that the values may only be considered as orientative ones. In most cases equilibrium was reached after 20-40 hours. An exception here were acetylmethionine and acetylleucine, for which the equilibrium was reached after several hours' incubation.

Control experiments, in which the acylase I preparation used was incubated with the various amino acids, but in the absence of the acetylating acids, showed that, unlike the enzyme employed by Greenstein [4], the present preparation was free from α-amine oxidase; a contamination with the latter enzyme would produce artifact results due to the decrease in the estimable α-amino nitrogen. The results of experiments dealing with the acetylation of various amino acids are presented in Table 1.

The data obtained in the experiments dealing with the enzymatic acylation of L-leucine by a variety of carboxylic acids are presented in Table 2. In order to ensure that equilibrium was reached in all cases, an additional amount of acylase I (0.3 mg/ml) was added to all incubation mixtures 20-40 hours after the commencement of incubation. The percentage of maximal synthesis and the equilibrium constant were calculated from the estimated amount of leucine at equilibrium.

Time-curves for the synthesis of some acyl derivatives of L-leucine are presented in Fig. 1.

DISCUSSION

It is obvious from the results obtained in the first experiment that the synthesis of the N-acetyl derivatives of most amino acids employed proceeded to a considerable degree; an exception in this case was aspartic acid, which was virtually not acetylated. The equilibrium constants, calculated for the various amino acids, varied from 0.06 for trytophane to 0.37 for methionine.

It is of interest that, while aspartic acid is not acetylated by acylase I, acetylasparagine synthesis proceeds to a considerable degree, although the reaction of acylase I-catalyzed hydrolysis the N-acyl derivatives of aspartic acid are not attached by the enzyme, while similar derivatives of asparagine are readily hydrolyzed. It would appear that the presence of a free β-carboxylic group in the substrate hinders, or even prevents its interaction with the enzyme.

TABLE 2

The Synthesis of Various Acyl Derivatives of L-Leucine in the Presence of Acylase I (The figures presented are mean values of 3-5 experiments, the variation not exceeding 5-7%)

Acylating acid		% acylleucine at equilibrium	equilibrium constant
name	Dissociation constant (published)		
Trichloroacetic	$2 \cdot 10^{-1}$	0	0
Monochloroacetic	$1.4 \cdot 10^{-3}$	5	0.02
α-Hydroxypropionic	$1.38 \cdot 10^{-4}$	9	0.04
Formic	$1.76 \cdot 10^{-4}$	11	0.05
Benzoic	$6.3 \cdot 10^{-5}$	13	—
Acetic	$1.75 \cdot 10^{-5}$	35	0.22
Propionic	$1.4 \cdot 10^{-5}$	40	0.27
Butyric	$1.48 \cdot 10^{-5}$	46	0.34
Valeric	$1.5 \cdot 10^{-5}$	<5*	—
Isobutyric	$1.5 \cdot 10^{-5}$	<4*	—
Isovaleric	$1.7 \cdot 10^{-5}$	0	—

*Because of the very low reaction velocity it is not possible to state with certainty that the equilibrium had been reached. The data quoted have been obtained after a 40-60 hour incubation period.

With respect to the net rate of enzymatic acetylation of the various amino acids, it would appear that the rates observed actually represent the difference between the true synthetic rate and that of the reverse reaction – the hydrolysis of the product. However, the experimentally observed initial rates were probably close approximations to the true values of the rate of synthesis, since in the initial reaction period the concentration of acylamino acids was low and therefore the rate of the reverse hydrolytic reaction could be expected to be insignificant. The initial velocities may therefore be taken to represent the true synthesis rate. The following points can be made from these observations:

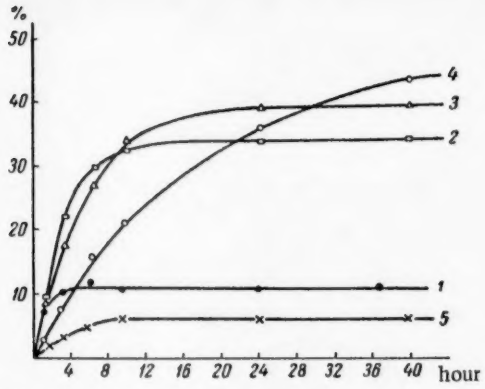
1. With aliphatic amino acids containing linear side chains of 2 and 3 carbon atoms (glycine, alanine), the rate of synthesis was approximately the same, comprising about 30 μ moles per hour per mg acylase nitrogen. With increasing length of side chain to 4-5 carbon atoms, the synthesis rate increased to about double the above value (68 for α-aminobutyric acid, 64 for norvaline), but with still longer side chains the rate decreased (35 for norleucine). It is of interest to note that in the case of the enzymatic hydrolysis of aluphatic N-acetylamino acids with linear side chains by acylase I the reaction rate increased steadily with the number of carbon atoms in the side chain, reaching a maximum with norvaline, and then decreased with further increase in the side chain, falling to 0 with α-aminododecanoic acid [11, 12].

2. The highest rate of enzymatic acetylation was observed in the case of methionine. It has been shown elsewhere [4] that the acylated derivatives of methionine are hydrolyzed by acylase faster than derivatives of any other amino acid.

3. Aromatic, dicarboxylic and dibasic amino acids were acetylated considerably slower than their aliphatic counterparts (phenylalanine – 20, glutamic acid – 15, tryptophane, ornithine, lysine – less than 5 μ moles per hour per mg acylase nitrogen). The same relationship was found previously to hold for the hydrolysis of the acetyl derivatives of the respective amino acids [5].

The results of the experiments on the enzymatic acylation of L-leucine by various carboxylic acids (cf. Fig. 1) show that the rate of synthesis decreased with the increasing number of carbon atoms in the aliphatic chain of the acid, the order of rates observed being: formyl > acetyl > propionyl > butyryl, which was the opposite to the reported [5-7] effect of the chain length on the hydrolysis of the corresponding acyl derivatives of amino acids by acylase I, where the reverse order held: formyl < acetyl < propionyl.

It will also be seen from Table 2, that there was virtually no enzymatic acylation of L-leucine in the



Time progress of the enzymatic synthesis of some acyl derivatives of L-Leucine. 1) Formyl-L-Leucine, 2) acetyl-L-leucine, 3) propionyl-L-leucine, 4) butyryl-L-leucine, 5) monochloro-acetyl-L-leucine.

presence of acylase I with the strong acid – trichloro-acetic. Acids of medium strength, with dissociation constants ranging from 10^{-3} to 10^{-4} (monochloroacetic, α-hydroxylpropionic and formic), were capable of some, though not intensive, acylation of L-leucine. At the same time some of the weaker acids, with dissociation constants in the region of 10^{-5} (acetic, propionic and butyric acids), acylated L-leucine to the extent of 35-45%.

However, the phenomenon of inverse relation between the acid strength and its ability to acylate L-leucine in the presence of acylase I, was by no means universal. It would appear that the process of L-leucine acylation in the presence of acylase I depended also on some factors other than the dissociation constant of the acylating acid. This, indeed, was borne out by the example of valeric acid, whose dissociation constant differed very little from that of butyric acid, while its ability to acylate L-leucine was so low, that only about 3-4% of the amino acid was converted to valeryl-L-leucine after 40-60 hours' incubation.

Similar divergences were observed when acids with branched chains were substituted for the straight-chain isomers. Thus, in the presence of butyric acid acylation proceeded to 46%, while with isobutyric acid the same reaction proceeded only to about 3-4%, and with isovaleric acid enzymatic acylation did not take place at all. Apparently, the actual acid strength of the acylating acid was no longer of prime importance, and the steric factors came into play; there may exist a definite limit for the volume of the radical which may interact, and, once this volume is exceeded, the steric hindrance greatly affects the reaction.

The above results are in agreement with published work of Fones and Lee [13], who studied the rates of hydrolysis of various acylamino acids by acylase I. These authors found a sharp decline in the hydrolysis rates in all cases when acylamino acids contained acyl radicals with spacially bulky side chains. It would appear that, both in the case of the enzymatic hydrolysis and synthesis of acylamino acids by acylase I, the increase of the acyl residue beyond a certain size interferes seriously in the interaction of the enzyme with the substrate.

SUMMARY

It was shown that in the synthesis of L-acylamino acids, acylase I was active towards a large number of L-amino acids. The extent of synthesis of acetyl derivatives was 20-40% with the majority of amino acids, and 48% with methionine.

The initial velocities, and the equilibrium constants for the enzymatic synthesis of 17 acetylamino acids have been calculated. The initial velocities of acylamino acid synthesis from various amino acids were found to bear approximately the same relation to each other as the corresponding initial velocities of their enzymatic hydrolysis.

The enzymatic synthesis of a variety of acyl derivatives of L-leucine from various carboxylic acids was studied.

The enzymatic acylation of L-leucine appears to be dependent on two factors: the strength of the acylating acid, and the volume of its side chain. In the case of acids with a straight side chain up to 4 carbons, an inverse relationship was found to hold between the dissociation constant and the ability to acylate L-leucine in the presence of acylase I.

Acids with side chains containing five or more carbon atoms, or containing branched side chains, were found to be either very poor substrates for acylase I in the acylation of L-leucine, or were completely unable to serve as substrates.

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THE EFFECT OF TETRAETHYLPYROPHOSPHATE ON THE ULTRAVIOLET ABSORPTION SPECTRUM OF PURIFIED ESTERASE

V. I. Rozengart and N. V. Kartasheva

Laboratory of Biochemistry, Institute of Toxicology, Academy of Medical Sciences, Leningrad

The spectrophotometric method of investigation has been used long and successfully for the study of protein structure. In recent years a number of studies have appeared in which the ultraviolet absorption spectra of proteins and individual amino acids have been studied at various pH values. In a number of cases, new data were thus obtained on the change in reactivity of individual functional groups in the protein molecule depending on external actions.

In 1943 Crammer and Neuberger [1] showed that as a result of denaturing proteins by urea there was a change in the character of the ultraviolet spectrum of protein solutions. In an alkaline medium the absorption maxima of such solutions shifted in the long-wave portion (from 275-280 to 295-300 mμ) and the coefficient of extinction rose markedly.

These results were fully confirmed by the work of Vendt and Tsyperovich [2], and also of Tonford and Roberts [3]. The further investigations of Vendt et al. [4] showed that with gradual change of pH from 6.0 to 12.0, in the majority of protein solutions the greatest increase in light absorption occurred at 248-253 mμ and also at 302 mμ. At 265-280 mμ there was no change at all, or a very slight one. The observed shifts could be related chiefly to ionization of the phenol group of tyrosine, since in pure tyrosine solutions an entirely analogous change was found. In solutions of gelatin, which contains almost no tyrosine, increase in pH did not change the light absorption.

Ungar et al. [5] used the increased optical density of protein solutions in an alkaline medium as a criterion of the partial denaturation of the protein. They succeeded in showing that a short electrical stimulation caused reversible denaturative changes in proteins of nerve tissue.

Thus, the possibility has been demonstrated of using the ultraviolet spectrophotometry for quantitative estimation of the slight structural changes of proteins which occur under the influence of external actions.

We decided to use this method to disclose the disruptions which the protein esterase undergoes when acted upon by the phosphoorganic insecticides.

It is known that many organic derivatives of phosphoric acid are powerful insecticides and at the same time very toxic for mammals. Their action can be explained to a considerable extent by the unique ability of these compounds in very small concentration to suppress the activity of choline esterase [6]. Liver esterase is no less sensitive to phosphoorganic insecticides than is choline esterase [7-9].

METHODS

Samples of purified liver esterase were prepared by the method worked out earlier in our laboratory [10]. The purified enzyme was dissolved in 0.9% NaCl solution to a concentration of 0.2-0.3 mg of protein per ml, and the ultraviolet absorption spectrum of this solution was measured on the SF-4 spectrophotometer. In each sample of protein the light absorption was measured at pH 7.0 and pH 12-12.5. Alkalinization was carried out

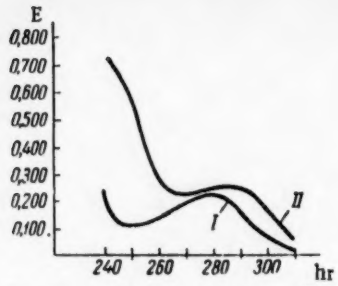

Fig. 1. Absorption spectrum of esterase solutions I) at pH 7; II) at pH 12.

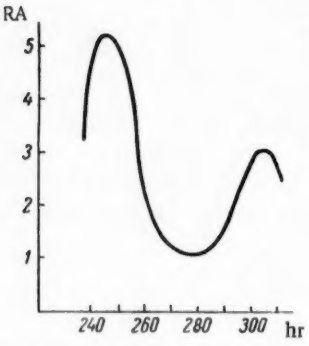

Fig. 2. RA curve of esterase solutions.

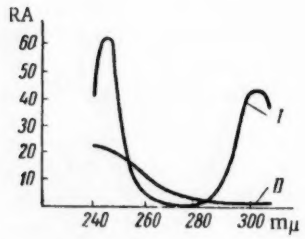

Fig. 3. RA curve for solutions of tyrosine (I) and cysteine (II).

with 1 N KOH and the final pH was determined with a pH meter with a glass electrode. Usually 0.2 ml of 1 N KOH had to be added to 10 ml of the protein solution to obtain the desired pH. In some of the experiments we took the full ultraviolet spectra from 240-310 mμ, but in most cases we limited the measurement of light absorption to that portion of the spectra in which the difference between the extinction at pH 12 and pH 7 was most clearly shown, that is, at 240-250 and 300-305 mμ. As a measure of the observed changes we took the ratio extinction at pH 12/extinction at pH 7 [5], which we called the "relative absorption" (RA).

Tetraethylpryophosphate (TEPP) was used as the example of the phosphoorganic insectides; this is known as a powerful inhibitor of choline esterase and liver esterase [9]. We used TEPP in a final concentration of 10^{-4} M which, as special experiments showed, was more than enough to suppress completely the activity of even the very concentrated esterase solutions which we used. Preliminary contact of the esterase with TEPP lasted 60 min at room temperature.

Denaturation of the protein was carried out by treating it with a 6 M urea solution for 2 hours.

RESULTS

In Fig. 1 we give a typical absorption spectrum of esterase solution at pH 7 and pH 12. The figure shows that the absorption spectrum of esterase in a neutral solution does not differ from that of the majority of proteins: it is characterized by a maximum at 275-280 mμ and a minimum at 250 mμ. In an alkaline medium, characteristic changes begin, in that the value of the extinction in the short-wave area of the spectrum rises sharply, the absorption maximum shifts to 290-295 mμ, and the mimimum to 270 mμ. These changes are analogous to those which have been described for some other proteins [2, 5]. The curve for RA calculated from these experiments is given in Fig. 2. Two sharply expressed maxima are characteristic for this curve: at 250 and 305 mμ, which shows that in just these parts of the spectrum there is the greatest difference between absorption in alkaline and neutral media.

These portions of the spectrum were chosen for study of the action of TEPP on esterase. Light absorption in solutions of the enzyme treated with TEPP and also in control solutions was measured at 240, 245, 250, 280, 300, and 305 mμ in neutral and alkaline solutions. Determination of the extinction at 280 mμ (at maximum absorption) served as a control and was used to determine the concentration of the protein in solution. For each wave length we calculated the RA value, the ratio of extinction at pH 12 to extinction at pH 7. The results are given in Table 1.

These data show that at all wave lengths except 280 mμ) the RA values for the solution of the enzyme treated with TEPP are lower than for the control solutions of pure esterase.

The presence of some scattering in individual results is produced because the determinations were carried out at different times and with different enzyme preparations; this forced us to estimate the differences more exactly by submitting them to statistical treatment by the method of Student-Fisher [11]. We showed that positively significant differences ($p < 0.02$) occurred only at 250 mμ. At 245 mμ the differences were at the border

of significance ($p \sim 0.05$), and at all the other wave lengths they were not significant.

Thus, the action of TEPP lowers the RA value of esterase at definite parts of the spectrum, that is, it causes a change in the structure of the protein molecule in contrast to what is found when some proteins are denatured. Since denaturative changes are not connected in all proteins with an increase in RA at 245-250 mμ, and for some proteins, such as trypsin [5], there is even found some decrease in RA in this part of the spectrum in denaturation, it becomes interesting to explain how denaturation acts on the liver-esterase protein.

TABLE 1

RA Values of Pure Solutions of Esterase (A) and of this Treatment with $1 \cdot 10^{-4}$ M TEPP (B)

A						B					
Wave length, mμ											
240	245	250	280	300	305	240	245	250	280	300	305
2,89	4,56	4,42	1,01	2,45	2,62	2,86	4,11	3,88	1,01	2,34	2,32
2,75	4,26	4,21	1,00	2,25	2,17	2,64	3,95	3,72	1,00	2,29	2,22
3,10	5,34	5,35	1,08	3,69	4,43	3,28	5,48	5,18	1,07	4,11	5,62
3,11	5,30	5,20	1,05	3,18	3,54	2,90	4,66	4,35	1,03	2,85	3,18
3,00	5,10	5,05	1,02	3,02	3,20	3,15	5,35	5,31	1,03	3,31	3,87
3,32	6,18	6,26	1,10	4,45	6,00	3,16	5,28	5,06	1,04	3,63	4,54
3,08	5,37	5,35	1,03	3,24	3,59	2,91	4,81	4,50	1,00	3,06	3,41
3,40	6,13	6,13	1,00	4,07	4,53	3,09	5,00	4,60	1,03	3,16	3,72
3,01	5,05	5,00	1,04	2,95	3,10	2,80	4,32	4,05	1,00	2,60	2,68
2,90	4,90	4,79	0,99	2,85	2,83	2,54	3,85	3,60	1,00	2,21	2,06
±,06 Average	5,22	5,18	1,03	3,22	3,00	2,93	4,68	4,42	1,02	2,96	3,36
Mean error ±0,14	±0,19	±0,18		±0,22	±0,34	±0,08	±0,19	±0,19		±0,17	±0,36
p						0,43	~0,05	<0,02		0,33	0,62

As the denaturing agent we used 6 M urea solution. For comparison we studied some other proteins along with esterase, proteins for which we found no data of this type in the literature. The protein solutions were denatured for two hours at room temperature, and then the light absorption was determined at pH 7 and pH 12.

Table 2, which presents the results of these experiments, shows that denaturation of all the proteins studied leads to definite increase in RA both at 245-250 mμ and at 300-305 mμ. By comparison of these results, with the fact of decreased RA of esterase under the influence of TEPP which we found, we can conclude first that TEPP does not cause denaturative changes in esterase solutions, and second, TEPP is actually bound to the functional group of the protein whose reactivity is increased by denaturation.

It is most probable that these groups will be found among the amino acids which make up the active centers of esterase, since it is known that phosphoorganic insecticides react selectively with that portion of the active surface of the enzyme which is responsible for the formation of the enzyme-substrate complex.

Analysis of the existing literature on the mechanism of action of the phosphoorganic compounds with the enzymes sensitive to them shows that there can be several parts to the active centers of the enzyme protein on which reaction can occur. Evidently the point of original attack of the phosphoorganic substances is the imino group of the imidazole ring which exists in the proteins as a histidine residue [12, 13]. Then the phosphorus-containing part of the insecticide is transferred to the hydroxyl of the serine residue and binds it firmly [14]. The phenol group of tyrosine also plays an important part in the active centers of esterase [15]. Therefore, we cannot exclude the possibility that the phosphoorganic insecticides also react with this amino acid in the molecule of enzyme protein. On the other hand, it is known that the ability for sharp change in light absorption with a shift of the medium to an alkaline pH is possessed by tyrosine [2, 4, 5] and cysteine [5].

TABLE 2

Effect of Denaturation by 6 M Urea on the RA Values of Solutions of Some Proteins. Action of Urea for 2 hours at Room Temperature

Protein studied	Wave length, mμ									
	245		250		280		300		305	
	N	D	N	D	N	D	N	D	N	D
Esterase	5.00	8.75	5.25	7.20	1.02	1.07	2.76	3.56	2.83	4.13
Cat serum	5.18	7.00	6.20	7.49	1.01	1.04	4.16	5.43	6.71	9.15
Rabbit myosin	3.80	10.4	3.36	5.86	1.10	1.03	1.90	3.10	1.58	3.18
Total globulin of rabbit serum	5.31	8.00	5.90	7.75	0.96	1.02	3.62	4.84	5.63	7.52

*N – native protein; D – denatured protein

Thus, there is a very small list of amino acids which can be considered as responsible for the changes which we have observed under the influence of TEPP. This list includes histidine, serine, tyrosine, and cysteine. In order to determine which of these amino acids must be given preference, we have, most importantly, determined the molar coefficient of extinction (ϵ) calculated for the amino acids at 245-250 mμ in an alkaline medium. The results are given in Table 3.

The table shows that among the amino acids studied only tyrosine and cysteine have a high enough value of ϵ so that change in the absorption spectrum of these amino acids can be reflected in the absorption spectra of dilute solutions of the proteins. In other words, the changes which we found under the influence of TEPP can be due only to tyrosine or cysteine. However, this does not exclude the possibility of reaction of TEPP also with histidine or serine, but if this produces a change, it cannot be observed under our conditions.

Figure 3 gives the values of RA for tyrosine and cysteine at different wave lengths. Comparison of these data with Fig. 2, which shows the RA curve of esterase, indicates complete agreement in character of RA curve of tyrosine and esterase; in both cases there is a definite maximum at 245-250 and 300-305 mμ and a minimum at 275-280 mμ. The RA curve of cysteine is characterized by a comparatively high value in the short-wave part of the spectrum which falls steeply at 245-270 mμ and then falls more gradually.

These results indicate that most probably the character of the esterase RA curve depends only on its tyrosine content. However, we cannot exclude the possibility that the maximum at 245-250 mμ is partly explained by ionization of cysteine, but this is less likely since the absolute RA value of cysteine and especially its molar coefficient of extinction ϵ are much lower than the corresponding values for tyrosine.

Therefore, we can believe that the decrease of esterase RA which we observed under the influence of TEPP is the result of its reaction with the phenol group of the tyrosine residues.

In a separate set of experiments we studied the effect of TEPP on a pure solution of tyrosine.

TEPP and tyrosine were mixed in equimolecular concentrations ($2 \cdot 10^{-4}$ M) and after incubation at room temperature for 60 min they were studied for optical density of their solution at pH 7.0 and 12, and their RA values were calculated. We introduced into the calculations a correction for the specific absorption of TEPP at each wave length.

As Table 4 shows, from the results of these experiments, as compared to those in which we used esterase solutions, TEPP has almost no effect on the RA of solutions of pure tyrosine. We could not run analogous experiments with cysteine since the low value of ϵ for cysteine required the use of higher concentrations of it ($1 \cdot \cdot 10^{-3}$ M), and TEPP at this concentration has such a high characteristic light absorption that it becomes impossible to carry out the measurements.

TABLE 3

Molar Coefficients of Extinction of Some Amino Acids in an Alkaline Medium (pH 12.0-12.5)

Amino acid	Wave length, mμ	
	245	250
Serine	9	8
Histidine	14	11
Cysteine	1550	1230
Tyrosine	9750	7000

TABLE 4

Effect of TEPP on RA Value of Tyrosine Solutions (Average data)

Wave length, mμ	Tyrosine	Tyrosine + TEPP
240	38.9	36.5
245	58.8	56.4
250	29.1	28.5
275	0.88	0.85
300	43.6	42.2
305	36.0	34.8

DISCUSSION

Our data indicate the ability of TEPP to react with the phenol group of tyrosine in the proteins of purified liver esterase. We did not attempt to study to what extent this reaction is connected with the inhibiting influence of TEPP on the enzymatic activity of the esterase, but some evidence of the possibility of such a connection can be shown on the basis of the existing literature data.

Recently Bergmann [16, 17] showed that the phenol group of tyrosine played a definite part in esterase catalysis. In his opinion, when the enzyme-substrate complex is formed, this group forms a hydrogen bond with the ether oxygen of the substrate and this assures further activation of the substrate molecule, which is very important since the basic activation which arises from nucleophilic reaction of the carbonyl carbon of the substrate with the imino group of the imidazole ring of the enzyme may be insufficient If this idea is correct, then any reaction which results in binding the phenol group of tyrosine should be able in some degree to sunpress the enzymatic activity.

The discrepancy between the data obtained with pure solutions of tyrosine and esterase solutions can perhaps be explained by the fact that in the composition of the protein molecule the functional groups of tyrosine are more reactive than in the free state.

An analogous increase in reactivity of special groups in proteins as compared to free amino acids is characteristic, for example, for the hydroxyl group of serine. Thus, after inactivation of liver esterase [14], choline esterase [8, 18], or trypsin [19] by diisopropylfluorophosphate labeled with P^{32}, phosphoserine-P^{32} could be isolated from the hydrolytic products of the proteins. At the same time, study of the direct action of some phosphoorganic compounds with solutions of the pure amino acid showed that neither serine, tyrosine, nor cysteine underwent this reaction [20]. It is true that the possibility was shown of the direct phosphorylation of the phenol group of tyrosine by tetraethylpyrophosphate under mild conditions (38°, pH 7-8), but this reaction went especially slowly and required almost a tenfold excess of TEPP [21].

The experiments described in this study do not permit us to exclude fully the possibility that the effect of TEPP which we found depends also on its reaction with the sulfhydryl group of cysteine. However, such a possibility is less likely in view of the literature data on the inability of TEPP and other phosphoorganic insecticides to inhibit such enzymes selectively (when their activity is connected with the presence of a free SH group [22, 23]) and hence the inability of TEPP in low concentrations to react with the sulfhydryl groups of proteins.

SUMMARY

For a study of the structural changes of the protein of purified liver esterase we used the method of determining the ultraviolet absorption spectra at different pH values. The observed changes were expressed by the ratio of extinction at pH 12 to extinction at pH 7 for each wave length used. These values were called the "relative absorption" (RA).

Under ordinary conditions, the RA curve of the solutions of purified esterase is characterized by two maxima (at 245-250 mμ and 300-305 mμ), and a minimum at 280 mμ.

We showed that treatment of the solution of esterase by tetraethylpyrophosphate at concentrations of $1 \cdot 10^{-4}$ M led to significant reduction in the value of RA at 245-250 mμ. These changes are not related to denaturation of the protein since denaturation of esterases and also myosin, serum globulin, and total serum proteins by 6 M urea was accompanied by a distinct rise in RA at 245-250 and 300-305 mμ.

On the basis of determination of molar coefficients of extinction of different amino acids and also their absorption spectra at different pH values of the medium, we can conclude that the responsibility for the observed changes in the esterase protein is in tyrosine and hence, that tetraethylpyrophosphate has the ability to react with the phenol group of tyrosine in the esterase molecule.

With pure solutions of tyrosine such a reaction does not occur.

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THE EFFECT OF IONIZING RADIATION ON THE ACTIVITY OF BONE-MARROW NUCLEIC ACID DEPOLYMERASES

R. E. Libinzon

Beginning with the work carried out under the direction of Hevesy [1-3] it has become well known that ionizing radiation causes a great change in quantity and metabolism of the nucleic acids of mammalian tissue. We showed in our earlier studies [4, 5] that the amount of desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) of bone marrow decreases sharply in the first hour after a single strong irradiation (dose 1000 r). It is difficult to explain this fall in amount of nucleic acids only by the inhibition of the synthesis of these compounds. We have suggested the idea that the observed fall in content of DNA and RNA is the result of the action of two opposing processes: a decrease in synthesis of the nucleic acids and an increase in their splitting. To test this idea we have undertaken to study nucleic acid depolymerase activity in bone marrow as the enzyme which first determines the splitting.

METHODS

The work was carried out on 19 controls and 50 rabbits (Shipshilla variety) given a single irradiation with a dose of 1000 r (γ-ray Co^{60}, strength of dose 15 r/min).

The basis for the process of determining the activity of the enzymes was the method of Schneider and Hogeboom [6]. The activity of ribonuclease (RNA-ase) was studied at pH 5.9 (0.02 M succinate buffer) and pH 8.2 (0.05 M borax + 0.1 M acetic acid) at substrate concentrations 0.5 and 2 mg RNA per ml. As the substrate we used twice-reprecipitated commercial RNA. The activity of the desoxyribonuclease (DNA-ase) was determined at pH 5.0 (0.1 M acetate buffer) and at pH 7.0 (0.05 M borate buffer)at concentrations of substrate of 2 mg DNA per ml of reaction mixture. As the substrate, we used highly polymerized pure DNA obtained from calf thyroid by the method of Gulland [7].

The source of the enzyme was an 0.5% homogenate of bone marrow added to the reaction mixture in the amount of 0.5-1.0 ml. $MgSO_4$ was added in the amount of 0.0125 M as activator. The reaction mixture (4 ml) was incubated for 60 min in a thermostat at 37°. The enzymatic process was stopped by adding 3% perchloric acid and 0.25% uranyl acetate. After standing for 30 min, the samples were filtered. The filtrate was diluted 12.5 times and the ultraviolet absorption was determined in it at 260 mμ on an SF-4 spectrophotometer. The controls were samples of the same composition, but the substrate was added after precipitation. Polarographic and fluoroscopic determination of uranium in the control and experimental samples did not show any difference.

The results are given in the form of increase of extinction per mg of homogenate nitrogen. All the data were subjected to statistical treatment and are given in the form of arithmetical average. The difference between experimental and control groups is considered real at $p \leq 0.05$.

RESULTS

The results of the investigations demonstrate the presence in bone-marrow tissue of two ribonucleases.

The optimum action of acid RNA-ase is produced at pH 5.9-6.0; of the alkaline enzyme at pH 7.6-8.3

[6, 8, 9]. The optimum concentration of RNA for bone marrow of normal rabbits, is, at pH 5.9, 0.5 mg/ml, and shows no particular difference at pH 8.2 with 0.5 and 2 mg/ml.

A single massive irradiation (1000 r) caused a considerable rise in activity of acid and alkaline RNA-ase. Thus, the enzymatic activity of the whole bone marrow homogenate, studied at pH 5.9, more than doubled ten minutes after irradiation at a substrate concentation of 0.5 mg/ml, and at a concentration of 2 mg/ml, rose 5.5 times (Table). The activity of the alkaline ribonuclease (studied at pH 8.2) at concentations of 2 mg/ml rose three times. Hence, in irradiated animals the substrate optimum for acid RNA-ase is shifted, and for alkaline RNA-ase this optimum is clearly shown. At later periods of radiation sickness (4, 12 and 24 hours, 2, 6 and 13 days) the activity of both acid and alkaline RNA-ase was appreciably increased. The highest activity of acid RNA-ase occurred after a day, of the alkaline RNA-ase, after 12 hours from the irradiation. Beginning on the second day we observed some decrease in ribonuclease activity, which, however, after 6 and 13 days remained considerably above normal.

We should note a second rise in enzyme activity of the alkaline ribonuclease 13 days after irradiation, when there is an increased rate of synthesis of RNA and DNA and regeneration appears.

The study of desoxribonucleases in bone marrow also shows the presence of two enzymes which split DNA: DNA-ase I has an optimum action at pH 7.0, requires the presence of magnesium ion, and evidently is close in properties to the DNA-ase of the pancreas. DNA-ase II is more active at pH 5.0-5.3. It is interesting that the enzymatic activity of this enzyme is clearly stimulated in the presence of 0.0125 M $MgSO_4$ which distinguishes the DNA-ase II of bone marrow from the acid DNA-ase of the thyroid gland [10, 11]. The enzyme has its greatest activity at a concentration of 3 mg/ml in the reaction mixture, but because of the difficulty of working with very viscous solutions, and for economy of the substrate, all the studies were carried out at a concentration of 2 mg/ml. We did not observe any quantitative difference in activity of acid and neutral DNA-ases.

Irradiation caused a clear increase in the activity of DNA-ase I four hours after irradiation (182% of the corresponding result for the control animals). A considerable increase in the activity of this enzyme was observed during the whole period of radiation sickness. The maximum rise on the first day of the sickness was after 12 and 48 hours (226 and 231% respectively); after 13 days, the activity of the enzyme was almost 3 times the normal.

The activity of DNA-ase II also rose after radiation, but the increased ability of bone-marrow homogenate to split DNA at pH 5.0 was somewhat less than at pH 7.0. At 24 hours after irradiation the activity of the enzyme reached 189%, and remained at a high level up to the 13th day (198%), with only a slight fall 6 days after irradiation (160%).

DISCUSSION

This investigation has shown the presence of active ribo- and desoxyribonucleases in bone-marrow cells of the rabbit. These results confirm the data of Brody and Thorell [12] on the presence of DNA-ase II and acid RNA-ase in chick bone marrow.

We did not observe any actual quantitative difference in the activity of acid and alkaline RNA-ase. We should emphasize that the acid RNA-ase of normal animals is more active at lower substrate concentrations: the optimum substrate concentration at pH 8.2 has not been observed. These results differ somewhat from those found by Lamirande et al. [9] who noted that for optimum activity, alkaline liver RNA-ase required 4.6 times more substrate than did the acid enzyme. We next consider the similar ability of rabbit bone-marrow homogenate to split DNA at pH 5.0 and 7.0. The activity of RNA-ase in rabbit bone marrow is only a little greater than the activity of desoxyribonuclease (1.17-1.3 times more), while in the same tissue of the chick this ratio is closer to 2-2.5. [12] and in mouse liver is 7-8 [6].

Brody and Thorell [12] observed a variable sensitivity of DNA-ase II to magnesium ions depending on the pH of the medium; at pH 5.2 the greatest activity of homogenate from chick bone marrow occurred in the presence of 0.1 M $MgSO_4$. According to our findings, also, the activity of DNA-ase II (pH 5.0) from rabbit bone marrow was higher in the presence of 0.0125 M Mg^{++} in the sample.

We are able to conclude from the results on change in nuclease activity under the action of ionizing radiation that the ability of bone-marrow homogenates to split RNA and DNA rises sharply after irradiation. The

Effect of Ionizing Radiation on Activity of Bone Marrow Depolymerases(Results Given as Average Values of Extinction, Calculated per mg of Homogenate Nitrogen and the Corresponding Error of the Arithmetical Average)

Time after irradiation	n	Ribonucleases						n	Desoxyribonucleases	
		acid pH 5.9		alkaline pH 8.2					ДНК,	
		RNA concentration, mg/ml							DNA-ase II, pH 5.0	DNA-ase I, pH 7.0
		0.5	2.0	0.5		2.0				
Control	19	69.5±6.9	32.7±3.7	65.1±6.3		60.1±6.5		10	45.8±5.4	51.4±11.7
10 min	7	160.7±19.6	177.0±17.4	103.0±17.3		190.0±75.4**		8	62.7±6.7**	58.6±4.8*
4 hours	5	110.4±12.7	179.9±14.9	61.1±4.6*		162.2±0.7		7	52.5±7.8*	93.9±13.2
12 »	7	143.0±11.0	171.0±14.9	87.5±6.6		202.9±14.6		7	71.2±3.7	116.0±5.7
24 »	5	100.6±24.3	231.3±24.0	82.0±11.2*		136.9±18.5		6	85.7±8.7	99.6±11.5
2 days	9	155.4±17.9	152.3±12.9	76.5±10.7*		127.3±14.3		7	80.6±9.6	118.6±6.3
6 »	7	105.8±3.5	116.8±13.9	67.2±5.0*		116.3±7.7		8	73.1±7.7	86.5±8.3
13 »	9	98.6±9.7	121.2±16.6	83.4±8.8*		190.0±17.1		7	90.7±15.7	150.2±23.3

*Difference between average data of control and experimental groups did not occur ($p > 0.1$)

**Probably a real difference between averages of control and experimental groups occurred $0.1 > p > 0.05$

activity of RNA-ase shows this rise more considerably than does that of DNA-ase.

Roth and Eichel [13] showed an increased ribonuclease activity in spleen homogenate of almost double after 16 hours from irradiation of rats. The rise in ribonuclease activity of liver homogenates was less constant [14,15]. There is evidence in the literature for an increased activity of DNA-ase II in spleen and thyroid [16,17], and the appearance of this enzyme has been observed in blood serum and urine of irradiated animals [18,19]. The increased activity of neutral DNA-ase in the spleen has not been described and there is also no evident increase in activity of acid and neutral DNA-ase in the liver of irradiated animals [20].

From all the above we can conclude that irradiation causes an increased ability to split DNA and RNA in homogenates of tissues with intensely proliferating processes. However, the reason for the activation of these enzymatic processes is not yet clear. When we analyze the possible mechanisms of this effect we must consider the probable change in localization of the enzyme within the cell. Thus, Roth et al. [13] showed that 37% of the RNA-ase activity of the spleen in unirradiated animals is connected with the mitochondria 28% with the hyaloplasm, but 68 hours after irradiation, only 15% is connected with the mitochondria, and 66% with the hyaloplasm. There is a fall of almost 3.5 times in the relative content of the enzyme in the nucleus [13].

We can assume that the increased content of the enzyme in the hyaloplasm and its decrease in the cellular organoids will make contact of the substrate and enzyme easier. The destruction of the mitochondrial structures may be the reason for this redistribution [21]. A second possibility to explain the increased depolymerase activity after irradiation is the probable destruction of inhibitors of the enzymes.

The presence of alkaline ribonuclease inhibitors in most tissues has been shown conclusively by Roth [22]. Some hindrance to the activity of alkaline ribonuclease inhibitors after irradiation was demonstrated as early as 1956 [15]. Later work by this investigator [23] on the study of the nature and properties of ribonuclease inhibitor permitted assumption that active radicals formed under the influence of ionizing radiation change the inactive form of the enzyme to the

active form by inactivation of SH-group inhibitors. Even less clear at the present time is the mechanism of increased activity of the desoxyribonucleases. It is possible that this process is also connected with the destruction of a protein inhibitor which was first found in the thyroid gland by a group of investigators under the direction of Laskowski [24]; the inhibitor was later found in many human and animal tissues [25]. Decrease in activity of DNA-ase I inhibitor in the spleen after irradiation was observed by Kurnick et al. [26]. The attempts of these investigators to detect the acid DNA-ase inhibitor have not yet met with success. Probably, the solution of a considerable amount of the enzyme as a result of destruction of mitochondrial structures also is of definite importance in the mechanism of increasing the enzyme activity. To explain the mechanism of nucleic acid depolymerase activation after irradiation certainly requires further experimental investigations.

We must also discuss the question of the biological significance of the increased activity of bone-marrow nucleic acid depolymerases after a single massive irradiation. As we mentioned above, we have shown [4, 5] that after irradiation there is a sharp fall in the amount of bone-marrow nucleic acids. Thus, the content of DNA and RNA four hours after the action of γ-rays (1000 r) is almost halved. A study of the rate of renewal of nucleic acid phosphorus in these animals, using P^{32}, established a simultaneous decrease in specific activity of DNA phosphorus of 49%, and of RNA phosphorus of 46%. However, since in a normal animal only about half the bone-marrow DNA is metabolized in a day (55% according to calculations made on the basis of our earlier data [27]), then in four hours there should again be synthesized only 9-10%, and the halving of the specific activity of DNA leads us to believe that the deficit of DNA resulting from hindering the synthesis will be only 4.5-5%. The loss of the remaining 44% remains unexplained.

The results of our study allow us to conclude that during the first day after irradiation, the DNA-ase activity almost doubles, which permits a considerable loss of DNA in the first hours after the action of ionizing radiation from the increased activity of DNA-ase I and II. The over-all effect of increased splitting and inhibition of synthesis explains only part of the loss; we believe that the rupture of hydrogen bonds and depolymerization of DNA which result from the direct action of the ionizing radiation and the action of products of the radiolysis of water make the splitting of DNA by the activated enzymes considerably easier.

A consideration of the rate of synthesis and enzymatic splitting of RNA shows that if we take the rate of renewal of RNA calculated on the basis of the experimental data, for 58 hours, then the decrease due directly to the suppression of synthesis is only 3-4%. The tripling of enzyme activity on a day (acid and alkaline ribonucleases at substrate concentration of 2 mg/ml) can to a considerable degree explain the loss in RNA content four hours after irradiation as coming from enzymatic splitting.

When we analyze the reasons for the fall in nucleic acids in bone marrow after irradiation we must certainly also consider the decreased number of cells in a unit weight of tissue in this period.

A considerable rise in nucleic acid depolymerase activity during two weeks after irradiation helps us to understand the fact that in spite of the great increase in their synthesis, which begins on the third day, the quantity of nucleic acids remains much below normal [4, 5]. On the other hand, the additional increase in activity of DNA-ase I and the alkaline ribonucleases 13 days after irradiation correlates with the great hastening of the renewal of nucleic acids (about 2.5 times) and the intensive regeneration at this time. The agreement in the times of strengthening of these processes probably does not lack definite biological significance.

Brody and Thorell [12] found a considerable rise in RNA-ase and DNA-ase activity in the period of intensive regenerative processes in chick bone marrow after injection of phenyl hydrazine. Allfrey and Mirsky [10] also assume that there is a connection between concentration of DNA-ase in the tissues and their ability to proliferate.

SUMMARY

We have detected the following nucleic acid depolymerases in the bone marrow of normal rabbits: acid and alkaline ribonucleases, acid and neutral desoxyribonucleases.

After a single irradiation at a dose of 1000 r we have studied the activity of the bone-marrow nucleic acid depolymerases after 10 min, 4, 12, and 24 hours, and 2, 6, and 13 days.

The activity of acid and alkaline ribonucleases increases three to five times even in 10 min after irradiation and remains high for two weeks after irradiation. The activity of the acid and neutral desoxyribonucleases

also rises in the first hour after irradiation and is increased one and a half to two times during the whole period of study.

Discussion of possible mechanisms of increased depolymerase activity after irradiation has been given, and it is suggested that there is significance in the observed effects of loss of nucleic acids.

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THE STRUCTURE OF GLOBULAR PROTEINS AND THEIR REACTIONS WITH THE EXTERNAL ENVIRONMENT

S. E. Bresler, V. P. Kushner and S. Ya. Frenkel'

Institute of High Molecular Weight Compounds, Academy of Sciences, USSR, Leningrad

In 1944, Bresler and Talmud [1] proposed a theory of the structure of globular proteins which considered them as formed from an equilibrium of three types of molecular forces: electrostatic repulsion of dissociated ionogenic groups, Van der Waals forces of uncharged side groups, and hydrogen bonds between peptide groups in the chains of principal valences. In order to estimate the energy of all three types of bonds, they took a simple model of the globulin with the chain coiled in a spiral ball and the hydrophobic (hydrocarbon) amino acid residues inside the globules where they formed, as it were, droplets of hydrocarbons. This idea has retained its value until now, but the model of the protein macromolecule has changed greatly.

According to the terminology introduced by Linderstrom-Lang, we meet with three types of structure in such complex polymers, structures which successively overlap each other as there is change from the more intense molecular forces to the weaker ones. The primary structure is formed from covalent bonds in the polypeptide chain (or several chains) of which the protein consists. The secondary structure is formed by the intramolecular hydrogen bonds between the successive peptide groups of the chain. The hydrogen bonds lead to coiling of the chain of chief valences into the α-spiral of Pauling-Corey. In such a spiral there exists the maximum possible number of H bonds between the peptide groups. However, the α-spiral is still not the final configuration of the globule. Thus, for example, if the macromolecule of serum albumin formed one α-spiral, then at a total number of 588 amino acid residues there would be a rod length of $588 \times 1.47 = 865$ A at a spiral diameter of 15 A, that is, the ratio of the axes would be $a/b = 58$. From the experimental data we know that the serum albumin macromolecule in the not-too-extended form has an axis ratio of only $a/b = 4.0$.

The tertiary protein structure is a somewhat compact packing of the δ-spirals into the form of a comparatively symmetrical body, the globule. The forces which act in the formation of the tertiary structure are the Van der Waals binding of un-ionized side radicals and the repulsions of similarly charged functional groups (except at the isoelectric point, the macromolecule always has an excess of charges of one or the other sign). These forces were previously studied by one of us [2]. However, they are not the only determinants of the tertiary structure of the globular macromolecule. The great variation in the tertiary structures of proteins arises from the formation of chemical disulfide bonds between separated amino acid groups in the chain. The disulfide bonds produce a special vulcanized tertiary structure of the protein, that is, they fix it by a special network of covalent bonds. In human serum albumin there are 17 such bonds. As chemical studies have shown [3-5] when the disulfide bonds of the protein are split by special reagents (reductive or oxidative), the tertiary structure of the protein, fixed by the vulcanization, is not at all held in equilibrium by weaker reactions (Van der Waals and electrostatic forces). In other words, the disulfide bonds fix the tertiary structure of the globule in a somewhat strained state. The nature of the geometrical packing of the α-spiral within the globule can be studied directly by X-ray methods. The spatial model found for myoglobulin by Kendrew shows topological complexities and irregularities in the organization of the tertiary structure of this protein. It is important to note that the disulfide bond is not the only form of cross bridging found in proteins. The same function can be performed by the ester link between side carboxyl groups of the dicarboxylic acids and the phenol hydroxyl of tyrosine, by the phosphate ester bridges, and also by salts formed by polyvalent metal ions with the side carboxyl groups of the proteins. We have studied the significance of the latter [5].

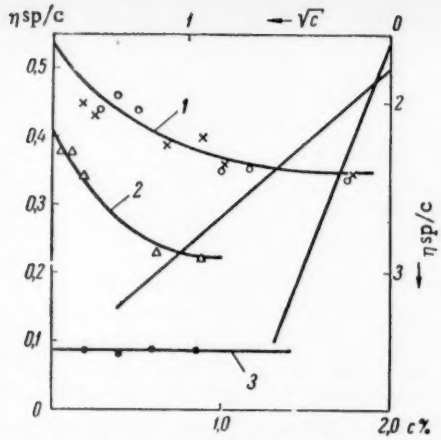

Fig. 1. Relation of viscosity to concentration for modified HuSA. 1) in borate buffer, pH 9.4; 2) the same + 50% dioxane; 3) the same + 0.3 M sodium acetate.

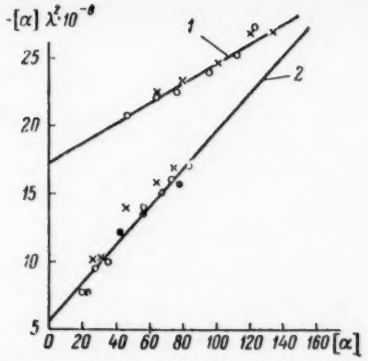

Fig. 2. Dispersion of optical activity of modified HuSA at pH 9.4. 1) in borate buffer; 2) the same + 50% dioxane.

TABLE 1

Some Characteristics of Modified HuSA

Protein	$-[\alpha]_D^{20}$	λ_C	K·10⁻⁴	[η]	Extent of spirals, %	
					from $[\alpha]_D$	from λ_C
HuSA, native, in borate buffer, pH 9.5, μ 0.1	57.9	272	16.0	0.04	32	43
HuSA, modified, same conditions	64.5	274	17.4	0.556*	26	45
HuSA, modified, in presence of 50% DO	29.0	382	5.8	0.003*	61	100**
HuSA, modified, in presence of 50% DO and 2% sodium acetate	29.0	382	5.8	0.083	61	100**

*By the formula of Fuoss.

** This simply means that at > 60% spirals the binomial equation is no longer obeyed.

The tertiary and secondary protein structures are not independent of each other. The compact packing of the α-spiral in the globule requires much bending. This leads to disorganization of the α-spiral at the point of bending and destruction of the oriented secondary structure. As a result of important theoretical [6-10] and experimental work [11-15] in recent years, methods have been worked out for quantitative estimation of the degree of existence of α-spirals in the packed polypeptide chains of synthetic polypeptides and proteins from the value of the optical activity in polymer solution and from dispersion of optical activity. The degree of presence of spirals in the polypeptide chains of native proteins is on the order of 40-60%. Hence, about half the extended polypeptide chains are in the disordered "amorphous" state. As has been shown [16], the inclusion of proline and hydroxyproline plays an important part in formation of the "amorphous" chain since they do not permit the formation of axial hydrogen bonds and packing in α-spirals. The tertiary structure also has an amorphous forming effect, especially the disulfide bonds. In a number of cases their destruction led to a considerable strengthening of the regular secondary protein structure [4].

In the present work we have set ourselves the problem of continuing the study of the tertiary structure of proteins, the molecular forces which create it, the interactions of the secondary and tertiary structures, and also

the special question of the role of the bridging disulfides first, and then of the tensions which they set up in the protein globule.

Naturally,we tried to use as the objects of our study two very different groups of proteins: 1) those which contain a considerable number of vulcanizing bridges which fasten members of the chain far removed from each other (we call these structural or supporting S-S bonds), and 2) those which are not at all "vulcanized" or which contain disulfide bridges only between nearby members of the chain, that is, which do not fix the tertiary structure (we usually call these local S-S bonds). This classification of S-S bonds was first given in publication [5]. Serum albumin belongs to the first group of proteins, and egg albumin and γ-globulin to the second.

The basic experimental method used in our work was the optical measurement of the degree of presence of α-spirals of the polypeptide chain and the study of the form and dimensions of the macromolecule by its hydrodynamic characteristics (sedimentation, diffusion, characteristic viscosity) under conditions: a) of systematic and smooth change in the properties of the medium, that is, solvents with change in pH and ionic strength, and also with solvents in which the composition is changed by full or partial replacement of water by organic liquids (dioxane, ethylene chlorohydrin, dimethyl formamide) which are much less polar than water and more likely to solvate the hydrophobic hydrocarbon groups of the protein; in some cases we added a special agent which breaks intramolecular hydrogen bonds, urea; and b) by chemical modification of the protein, that is, by irreversible action of different agents which selectively attack one or another group in the protein.

In the past, these methods were partially used, mostly for the study of synthetic models of polypeptides [17-19] and only in a few cases for the study of proteins [5, 14-15, 20].

A third direction of study was the search among the proteins for a sharp temperature break of the type of break between order and disorder found by Doty in model polypeptides which he called intramolecular melting. The great extent of the oriented secondary structures in the protein and the cooperative nature of the interaction of amide groups in the α-spirals forces us to consider the secondary structure as an intramolecular crystallization, and hence justified a glance at the splitting of this structure in the phase transformation, the intramolecular melting.

The first results of the present work were reported by one of us at the Faraday discussion of April, 1958 [21].

EXPERIMENTAL*

Materials and Methods

In this work we used these proteins: HoSA and EA were crystallized by the method by Baranovskii [22]; γ-Gl was obtained by the method of McMeekin [23]; HuSA was a commercial preparation (dried lyophilically).

As solvents we used borate or citrate buffers with different pH values and ionic strengths 0.2 and 0.1, respectively, and also pure DO, ECH, and DMF.

Modification of the HuSA by MIA was carried out by boiling a 1% solution of the protein with MIA added to a final concentration of 15 μ moles/ml for 10 min in a borate buffer, pH 9.5 or 8.5, as was described previously [5]. Oxidation of HuSA by performic acid was carried out according to the description of Reichmann and Colvin [24]; the oxidized protein contained a considerable number of aggregates.

The concentration of the protein in the solutions was determined by the Kjeldahl method or from the dry residue. Determination of the change of degree of coiling in the globular macromolecule was carried out by measurement of the hydrodynamic properties: viscosity, in an ordinary Ostwald capillary viscosimeter at $20 \pm 0.01°$; sedimentation constant, in a Svedburg oil ultracentrifuge at 60 000 rpm, and diffusion constant in a Lamm apparatus. The results on sedimentation and diffusion were obtained at the standard temperature of 20° and in pure water. In some cases the molecular weight of the modified protein was determined by the method of light scattering.

* Hereafter we will use the following abbreviations: HuSA – human serum albumin, HoSA – horse serum albumin, EA – egg albumin, γ-Gl – horse γ-globulin, RNA-ase – ribonuclease, DO – dioxane, ECH – ethylene chlorohydrin, DMF – dimethyl formamide, MIA – sodium monoiodoacetate.

TABLE 2

Change in Form of the HuSa Macromolecule When it is Modified by MIA

Protein	[η]	a/b
Native	0.04	3.4
Modified HuSA	0.60	27.0

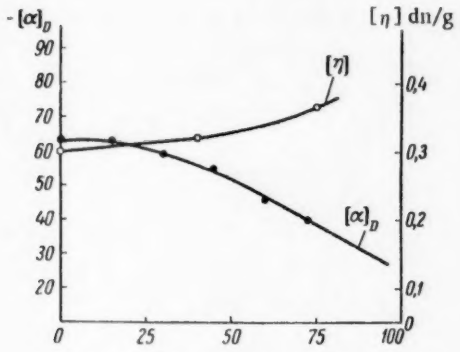

Fig. 3. Specific optical rotation ($[\alpha]_D$) and characteristic viscosity ($[\eta]$) of HuSA depending on dioxane content in a citrate buffer, pH 2.0.

TABLE 3

Spiral Formation of HuSa in the Presence of Dioxane in a Citrate Buffer, pH 2

Dioxane, %	Spiral formation from $[\alpha]_D$	Spiral formation from λ_C
0	27	29
15	27	31
30	31	34
45	35	45
60	44	45
72	50	40

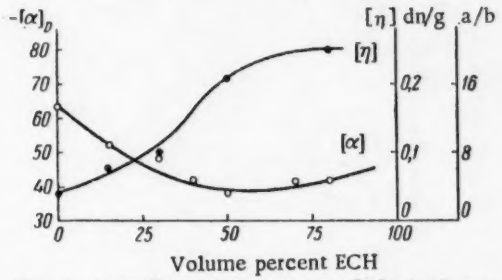

Fig. 4. Specific optical rotation ($[\alpha]_D$), characteristic viscosity ($[\eta]$) and axial ratio (a/b) of HuSA depending on content of ethylenechlorohydrin in a citrate buffer, pH 3.5. Axial ratio calculated on the assumption that the HuSA macromolecule has the form of an extended ellipsoid rotation with 20% hydration.

The degree of spiraling of the polypeptide chains was estimated by the value of specific optical rotation at definite wave length and temperature ($[\alpha]_D^{20}$ at λ = 5893 A, which corresponds to the D line of sodium at 20°). In a number of cases we also measured the optical rotatory dispersion in the visible region. Calculation of the dispersion constants (λ_C and K) and estimation of the percentage of spiraling by $[\alpha]_D$ and λ_C was carried out in accord with the method of Yang and Doty [14]. The refractometer correction, which in our case did not exceed 1-2%, was not made. All the optical rotation measurements were carried out on a precision polarimeter connected to a monochrometer. The relative error of measuring the angle of rotation was 1-2%. Measurement of $[\alpha]_D$ at temperatures from 10 to 95° was carried out in a special thermostat controlled cuvette.

RESULTS AND DISCUSSION

Change in Optical Activity and Hydrodynamic Properties of Modified HuSA

As we mentioned earlier, one of the possible methods of studying the intraglobular forces is to modify the macromolecular structure directly by the action of specific agents on definite functional groups of the protein. The agents which we chose were chiefly those which weakened the tertiary structure: breaking S-S bonds, stretching the globules by the forces of electrostatic repulsion of similarly charged groups, etc.

It is known [25] that as a result of the reaction of SH and NH_2 groups of proteins with MIA, they undergo carboxymethylation:

$$R{-}SH + CH_2ICOONa \rightarrow HJ + R{-}S{-}CH_2COONa$$

$$R{-}NH_2 + CH_2ICOONa \rightarrow HI + R{-}NH{-}CH_2COONa \xrightarrow{+CH_2ICOONa} \rightarrow RN(CH_2COONa)_2 + HI$$

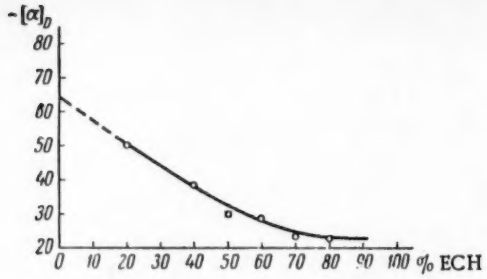

Fig. 5. Change in specific optical rotation ($[\alpha]_D$) depending on content of ethylene-chlorohydrin for HuSA oxidized by performic acid, pH 2.0

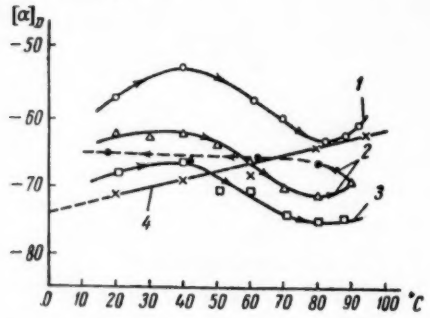

Fig. 6. Dependence of $[\alpha]_D$ on temperature; 1) HuSA in borate buffer, pH 9.4; 2) HuSA in borate buffer, pH 7.6; 3) HuSA in citrate buffer, pH 1.9; 4) oxidized HuSA in borate buffer, pH 9.4. In this figure, and also in Figs. 8, 9, and 10, the dotted parts of the curve mean reversed direction (cooling).

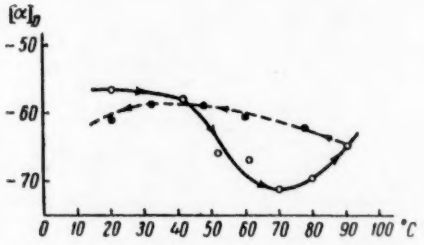

Fig. 7. Dependence of $[\alpha]_D$ on temperature: for HoSA in a borate buffer, pH 9.4.

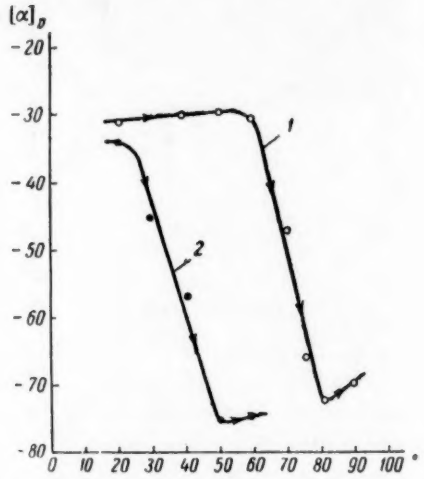

Fig. 8. Dependence of $[\alpha]_D$ on temperature: 1) EA in borate buffer, pH 9.4; 2) EA in citrate buffer, pH 1.8.

The completeness of carboxymethylation is measured by determining the amino groups by the Van Slyke method. The substitution of carboxyl groups for the amino groups leads to change in the electrostatic charge of the globule, which is shown especially clearly at an alkaline pH. Because of the electrostatic repulsion of the similarly charged groups (COO^-), the native configuration of the globule is destroyed and it is converted to the analogous linear polyelectrolyte. This was observed in the modification of HuSA when MIA was used.

In Fig. 1 we give the curves, typical for weakly ionized polyelectrolytes, of relation of viscosity to concentration. The straight lines shown in this graph are the result of calculating the data by the formula of Fuoss [26], which is valid for linear polyelectrolytes $\eta_{sp}/C = A/(1 + Bc^2)$. Extrapolation of these straight lines to zero concentration permits estimation of the value of the characteristic vsicosity.

Fig. 2 gives the results on dispersion of optical rotation in the form of a Doty graph, $[\alpha]\lambda^2 = f(-[\alpha])$, valid for the binomial equation of Moffitt [14]. The chief results of these experiments at pH 9.5 are given in Table 1.

Fig. 1 shows that with decreasing protein concentration in the solution there is a sharp increase in the viscosity. This shows that the macromolecule is more straightened at decreased concentration, since the electrolytic repulsion of the similarly charged groups is increased as a result of lower ionic strength in the solution and the related expansion of the screening atmosphere of opposite ions around each charge. This decrease in viscosity is not connected with the effect of aggregation, since the molecular weight after modification, measured by our method of light scattering, is exactly equal to the molecular weight of the original protein (70 000).

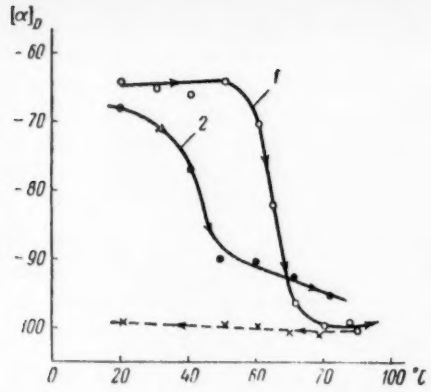

Fig. 9. Dependence of $[\alpha]_D$ on temperature: 1) γ-Gl in borate buffer, pH 9.4; 2) γ-Gl in citrate buffer, pH 9.4.

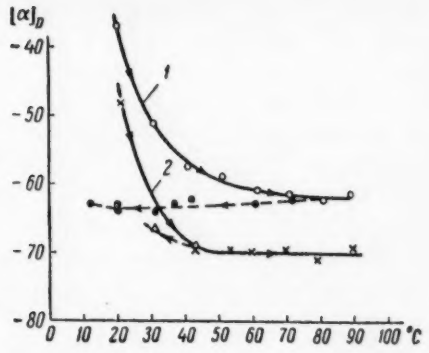

Fig. 10. Dependence of $[\alpha]_D$ on temperature: 1) EA in 30% dioxane mixture, pH 8.8 (ionic strength 0.01); 2) EA in 4 M solution of dimethylformamide in a borate buffer, pH 9.4.

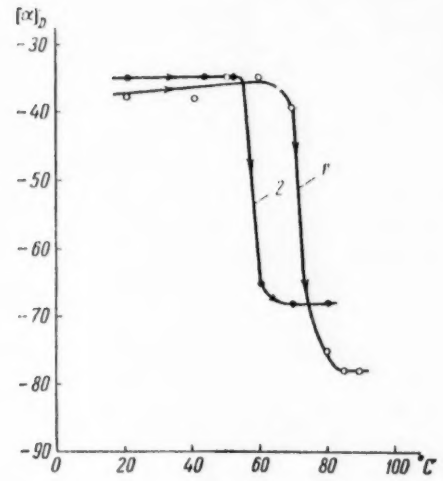

Fig. 11. Dependence of $[\alpha]_D$ on temperature: 1) EA in borate buffer, pH 9.4; 2) the same + 4 M urea.

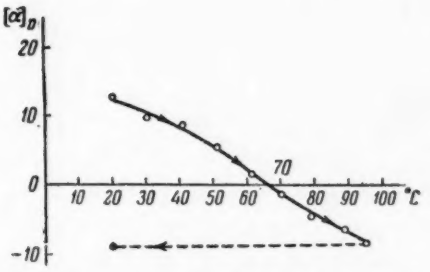

Fig. 12. Dependence of $[\alpha]_D$ on temperature for EA in 98% ethylenechlorohydrin. Dotted part of the curve is after cooling.

A most interesting result of the modification is the increased ability of the macromolecule to form spirals when there is partial replacement of the water by dioxane because of the weakening of the tertiary structure. In 50% DO-water mixture $[\alpha]_D^{20} = -29°$ and the corresponding degree of spiral formation is 61%, while the maximum percent of spiral formation for the original (native) HuSA in a DO-water mixture is reached at 20% DO and does not exceed 42% [21].

As would be expected, the polyelectrolyte effect is completely removed if some quantity (0.3 M) sodium acetate is added to the solution (lower line, Fig. 1), to create sufficient ionic strength. The increased spiral formation is retained.

Electrostatic forces between the ionized groups on the surface of the modified HuSA macromolecule stretch the macromolecule into a rod, but are not sufficient to rupture the hydrogen bonds within the α-spiral and convert it into a linear chain. The number of carboxyl groups on the surface of the modified HuSA (with complete carboxymethylation of all the amino and sulfhydryl groups) is of the order of 250 to 580 amino acid links in the chain. It is interesting to compare the behavior of the modified protein with the behavior of the model polymer, polyaspartic acid. In the polypeptide whose chain forms an ideal α-spiral, with replacement of the hydrogen of the carboxyl group by sodium there is an increase in charge [27] and only when the degree of dissociation reaches about 40% does there begin unfolding of the α-spiral and its transformation into a linear chain.

In order to determine the form of the macromolecule of the modified protein, we calculated the ratio of the semiaxes of the ellipsoid-rod a/b from the values of the characteristic viscosity, using the table of Mehl, Onkley, and Smith [28]. There still remained unknown the degree of the hydration of the protein, which must be considered in an analysis of the hydrodynamic characteristics. However, since the value for the hydration of the proteins, according to all data, does not exceed 20-30%, then all the corrections for hydration would not be more than 25% of the value of a/b and therefore would have little effect in the analysis of such large effects. We give values for a/b of native and modified HuSA on the assumption that the hydration equals 0.2 (Table 2).

We see that the macromolecule of modified protein is lengthened about 8 times as a result of the weakening of the tertiary structure, while it retains the spiral configuration of the chain of chief valences. In this respect the ratio of semiaxes a/b is half as great as in the α-spiral of 580 amino acid residues stretched to the limit.

Another method of modifying the protein macromolecule consists in partial or complete elimination of the S-S bonds. Experiments on denaturing under pressure [5] and experiments of Belitser et al. [3] on reversible reduction of the S-S bond in serum albumin have shown that with destruction of the structure of the S-S bonds there is a marked gain in the compact packing of the globule. Complete destruction of the S-S bonds is attained by oxidizing the protein with performic acid [24]. Then there is an important change in secondary structure. There are two sets of facts here, and two corresponding viewpoints. On the basis of experiments with RNA-ase [15], Harrington and Schellman agree that the α-spiral structure cannot exist in water solutions without the support of S-S bonds. They gave optical and hydrodynamic data which showed that by oxidation with performic acid, RNA-ase lost its spiral nature and went over to the state of a statistical ball. However, the limiting value for levorotation of oxidized RNA-ase obtained by Harrington and Schellman ($[\alpha]_D^{20} = -92°$) still does not correspond completely to a disordered structure of the macromolecule; thus, for example, in 8 M urea there is a further increase of $-[\alpha]_D^{20}$ to 108.5°.

Marcus and Karusch [4] came to another conclusion. On the basis of experiments with HuSA they showed that with full reduction of all S-S bonds in the protein its levorotation not only did not increase, but was even considerably decreased. This confirmed the strengthening of the α-spiral structure.

Our data on the measurement of $[\alpha]_D$ in oxidized HuSA, given in the next section, show that levorotation is almost unchanged by oxidation, but the oxidized protein has a considerably greater ability to form the α-spiral structure in a medium (solvent) favorable for this. Hence, we see that when the S-S bond is eliminated, there is a double effect: there may be a breakdown of some part of the α-spiral structure which is stabilized by these bonds, but at the same time the tensions created by these S-S bonds in the macromolecule disappear and under suitable conditions it can form spirals, acquiring a more perfect secondary structure.

Changes in Secondary and Tertiary Structures When Solvents are Varied

The experiments with addition of dioxane to water solutions of HuSA in an alkaline medium (pH 9.5-11.0) were carried out in our laboratory and have already been described [21]. The action of such mixed solvents can be treated as follows: as a result of the weakened Van der Waals forces between the hydrophobic groups of the side chains of the protein macromolecule, there is a stretching of the globule which is detected by the change in viscosity and sedimentation; second, there is a further spiral formation: levorotation is decreased.

These conclusions fully retain their force also for the acid region of the pH, as is evident from Figs. 3 and 4. On these graphs we give the changes which occur with HuSA on partial replacement of water by one of the slightly polar organic solvents (DO in Fig. 3 or ECH in Fig. 4). We see how both cause marked decrease in levorotation: $[\alpha]_D^{20}$ falls in both cases from -62° to -40° with change in concentration of DO and ECH respectively from 0 to 70-75%. This effect may be connected with supplementary α-spiral formation (Table 3) caused by disturbance in the tertiary structure. The supplementary spiral formation accompanies deformation (lengthening) of the macromolecule, particularly obvious in the presence of ECH (Fig. 4): the axial ratio a/b changes 4.0-4.5 times.

When the concentration of organic solvent is raised, we reach a limiting "saturated" α-spiral structure at which $[\alpha]_D^{20}$ and $[\eta]$ tend asymptotically to the values: $[\eta] = 0.27$ and $[\alpha]_D^{20} = -40°$. Such a limiting state in the case of HuSA (and also other serum albumins) is determined by the presence of the hard form of the globule which is composed of 17 S-S bonds, the majority of which are structural. The S-S bonds limit the mobility of

the portions of the macromolecule chain held between them and exclude the possibility of further spiral formation. Such a view of the role of the S-S bonds is confirmed by experiments with oxidation by performic acid for proteins in which the S-S bonds are eliminated. In Fig. 5 we give the curve of relation of $[\alpha]_D^{20}$ to concentration of ECH in the medium. In this case, the limit amount of spiral formation is markedly raised: in an 80% ECH mixture, $[\alpha]_D^{20} = -22°$ (instead of $[\alpha]_D^{20} = -40°$ in the native protein). The increased ability of the protein to form the secondary structure when the S-S bonds are eliminated is undoubted.

Transformations of the Type Order-Disorder in Globular Proteins

Investigations in recent years have shown that in synthetic polypeptides with variation in the solvent from neutral towards the formation of H bonds there begins a rather sharp transformation from the α-spiral chain structure to the statistical ball [14, 17]. This means that the splitting of the system of H bonds in the α-spiral occurs as a "cooperative effect" and resembles melting [29-31].

In proteins this fact is complicated by the presence of the tertiary structure and the structure of S-S bonds. The macromolecule consists of more or less perfect α-spiral portions and an amorphous field. Harrington and Schellman [15] found a faint similarity to melting in the secondary structure of RNA-ase close to 60°.

The transformation was very incomplete and prolonged at 20-30°. When we consider the number of retained S-S bonds, and the imperfections of the secondary structure of RNA-ase, such behavior of the protein is not surprising.

We obtained similar results for HuSA at pH 9.4 and 7.6 (borate buffer) and pH 1.9 (in a citrate buffer, Fig. 6). The intramolecular "melting" of another strongly vulcanized protein, HoSA, is shown in Fig. 7 (in a borate buffer, pH 9.4). Both graphs show that in the case of strongly vulcanized proteins the observed effect of "melting" is small; according to $[\alpha]_D$ it consists in all of 10-15°, which corresponds to a decrease in spiral formation from 28 to 18% (when HuSA melts in a borate buffer at pH 7.6), and is prolonged over the temperature range. The limiting value for levorotation for the melted secondary structure is -76° (14% spiral formation) and depends on the conditions of the experiment (for example, pH of the medium). This shows that in the given case not all of the α-spiral structure of the protein macromolecule is melted, but only the part of it connected with the presence of the structural S-S bonds. This is indicated by the absence of melting in the oxidized HuSA (Fig. 6). A second interesting fact is the irreversibility of the transformation. This is evident from the curves of Figs. 6 and 7.

An entirely different picture is obtained in the case of proteins which do not have structural S-S bonds. In Fig. 8 we give results on the effect of temperature on optical activity for EA at pH 9.4 and 1.8, and in Fig. 9 the same curves for γ-Gl. Here the effect of "melting" is greatly increased and consists already of 35-45° according to $[\alpha]_D$ (the spiral formation is changed from 60 to 18% when EA melts in a borate buffer, pH 9.4); at the same time there is an increase in temperature effect: the change is completed in an interval of 5-10°. The value of the optical activity is established quickly (2-3 min) when the solution is heated, but the melting curve is irreversible. Cooling the solution does not lead to reverse changes in optical activity.

In Figs. 8 and 9 the effect of pH is clearly evident: the "melting" curves are shifted toward lower temperatures when the pH is changed from 9.4 to 1.8.

It is interesting to see how the intramolecular "melting" occurs, if the method of varying the solvent is used on the tertiary and secondary protein structures. Experiments of this type are illustrated in Figs. 10, 11, and 12. In Fig. 10 we show the action of 30% DO (added to a borate buffer, pH 9.4) and a 4 M solution of DMF (in the same buffer).

We see that the weakening of the tertiary structure because of addition of nonpolar solvents causes labilization (but not a quantitative increase) of α-spiral structures. EA begins to melt much earlier, at a temperature of 25-30° instead of 70-80°. The 4 M urea shows a somewhat weaker action (Fig.11). In pure ECH (Fig. 12) the picture is again changed: we do not find "melting" at all up to 100°. In this solvent the α-spiral structure is very perfect and the hydrogen bonds are especially strong because of the absence of the competing action of water.

These experiments indicate several mechanisms for the action of solvents on proteins. The action of typical slightly polar solvents when compared to water, DO and DMF, destroys the tertiary structure and hastens

the "thermal destruction" of the secondary structure. The supporting action of the tertiary structures then stops and melting of the system of hydrogen bonds occurs at a lower temperature. Urea, on the other hand, is a specific solvent for just the secondary structure; when its concentration is high (8 M) splitting of the intramolecular H bonds should occur at room temperature. In our case 4 M urea eases the melting of the α-spirals, but not by acting on the tertiary structure; rather, directly. From this point of view, the phase transformation does not resemble the melting of pure substances, but of two component systems. This intramolecular cooperative effect is what we have chosen to call the order-disorder transformation.

Directly contrary to the action of urea is the action of anhydrous ECH which in itself weakly forms H bonds and therefore makes the secondary structure resistant to solvents. At the same time, ECH, like the remaining nonaqueous solvents, eliminates the tertiary structure ($[\eta]$ of EA in ECH 0.7 dl/g which corresponds to cylindrical particles with axial ratio about 30). This experiment shows that the melting of the α-spirals, in the absence of a water molecule to compete for the H bond, occurs above 100°.

Thus, from a consideration of the reactions of secondary and tertiary structures we can distinguish stabilizing and competing actions. On the one hand, the tertiary structure stabilizes the secondary, but this occurs because of a quantitative decrease in degree of spiral formation. On the other hand, weakening of the tertiary structure by slightly polar solvents, though it leads to a gain in degree of spiral formation, yet lowers the "heat stability" of the secondary structure.

SUMMARY

The experiments described in this paper show that the method of chemical modification of proteins and variation of solvents permits a detailed investigation of the reactions of secondary and tertiary structures of globular proteins. The greatest value is found with processes in which competition occurs between both structures. Such are processes of decreasing levorotation of proteins when water is replaced by slightly polar solvents, the considerable gain in spiral formation in MIA modification, etc. The idea of a state of tension of the globules because of the presence of structural S-S bonds in the macromolecule has obtained direct experimental confirmation in experiments with oxidized HuSA. Experiments on the study of "melting" of α-spirals show directly the existence in the globular proteins of a phase transformation in which there occurs a structural reorganization of the protein globule within the limits of the secondary and tertiary structures. A study of the effect of different factors on intramolecular melting permits us to explain both the nature of this phase transformation and the significance of the tertiary structures in maintaining the α-spiral configuration of the polypeptide chains. Fianlly, there is one further consequence of all this. It is evident that at the present time it is possible to delimit the different intramolecular changes which occur in the molecules of globular proteins. Under these conditions, the idea of denaturation loses all the physicochemical meaning which is based on any structural change of the protein which is not related to hydrolysis of the peptide link. From this point of view, we can speak concretely of the type of intramolecular reaction and the accompanying morphological changes, and the term "denaturation" can retain its customary sense of the loss by the protein of its functional activity as a result of external action.

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THE PRESERVATION OF TRYPTOPHAN WHEN PROTEINS ARE HYDROLYZED IN THE ATMOSPHERES OF SEVERAL GASES

Yu. N. Kremer, I. R. Meirovits and A. A. Shmidt

Department of Biological Chemistry, Medical Institute, Riga

In the study of the mechanism of action of sulfur dioxide as a factor which prevents the destruction of trypotphan when it reacts in a strongly acid medium with some aldehydes and sugars, it was shown that this effect is not specific, since other gases also (N_2, CO_2) under the same conditions show an analogous protective action. It was shown that the protective action of CO_2 is even more pronounced than the action of sulfur dioxide [1]. It is of interest to establish how much the protective action of these gases is shown in regard to tryptophan of proteins when they are hydrolyzed by strong acids, since it has been demonstrated that sulfur dioxide under these conditions prevents the destruction of tryptophan to some extent [2-4]. These results have been confirmed in recently published work [5].

METHODS

We used fully washed cattle fibrin for the study. The fibrin was digested by pepsin and the peptolyzate was treated with concentrated H_2SO_4 in the ratio peptolyzate / H_2SO_4 = 4/1 (by volume). The conventional protein content (N × 6.25) in the solution was 4.23%. Over a period of 9 hours separate portions of the solution were refluxed in the ordinary atmosphere and in an atmosphere of the following gases: CO_2, H_2, N_2, SO_2. Each 3 hours we took samples for determination of total nitrogen (by Kjeldahl), amino nitrogen (according to Pope and Stevens [6]) and tryptophan (according to Udenfriend and Peterson [7]). The gases were obtained by the usual methods and were purified from possible admixtures of oxygen and moisture by passing through solutions of pyrogallol and concentrated H_2SO_4. Passage of the gases was begun 20 min before beginning of boiling (for complete removal of air from the system) and was stopped only at the end of the hydrolysis. We ran two sets of experiments. The second series differed from the first in that hydrolysis was carried out with 10% hydrochloric acid.

RESULTS AND DISCUSSION

The data show conclusively that when protein hydrolysis is carried out with strong acids, an oxygen-free medium should be used for the preservation of tryptophan. Thus, for example (Fig. 1), when hydrolysis is carried out with 20% sulfuric acid for 3 hours in a CO_2 atmosphere, 90% of the tryptophan is preserved, in an atmosphere of hydrogen, nitrogen and sulfur dioxide, 65.5, 57.4, and 40%, respectively, while in an atmosphere of air, less than 20% of the tryptophan is preserved. After a 9-hour hydrolysis in a CO_2 atmosphere, 70% of the tryptophan is still preserved, while with the same length of hydrolysis in an atmosphere of air, tryptophan has been almost completely destroyed. It is not hard to see that, independent of all other conditions, a CO_2 atmosphere, as a rule, preserves tryptophan most fully. This agrees completely with the earlier data [1] which showed that CO_2 greatly prevented the destruction of tryptophan when it was boiled with 20% sulfuric acid in the presence of substances which destroy tryptophan (sugars, aldehydes), more so even than such gases as nitrogen and sulfur dioxide.

On the basis of these results, all the gases studied can be arranged in the following order of decreasing protective action: $CO_2 > H_2 > N_2 > SO_2$. Thus, we have confirmed our earlier opinion [1] of the nonspecific pro-

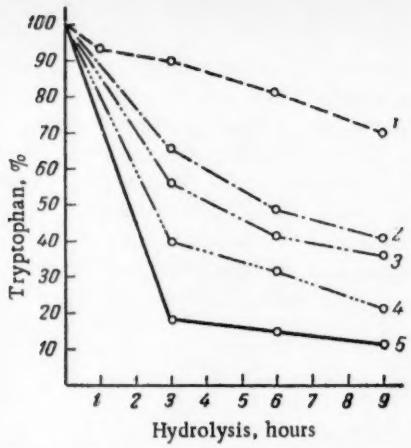

Fig. 1. Preservation of tryptophan in fibrin hydrolysis by 20% sulfuric acid in an atmosphere of: 1) CO_2; 2) H_2; 3) N_2; 4) SO_2; 5) air.

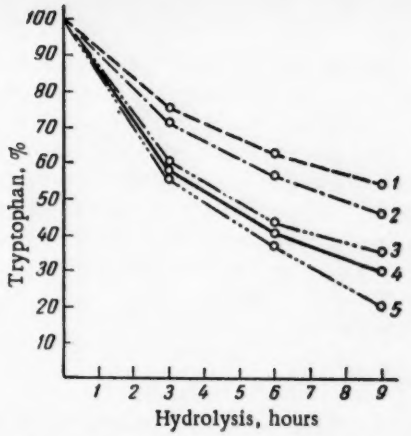

Fig. 2. Preservation of tryptophan in fibrin hydrolysis by 10% hydrochloric acid in an atmosphere of: 1) CO_2; 2) H_2; 3) N_2; 4) SO_2; 5) air.

Extent of Hydrolysis in an Atmosphere of Some Gases in Relation to Its Duration. In % of Amino Nitrogen Based on Total Nitrogen of the Hydrolyzate

Hydrolysis, hours	20% sulfuric acid					10% hydrochloric acid				
	air	CO_2	H_2	N_2	SO_2	air	CO_2	H_2	N_2	SO_2
1		41.4								
3	54.5	54.0	60.8	59.1	59.1	58.3	58.4	57.1	56.4	54.4
6	57.0	55.1	61.9	61.5	59.2	64.5	62.0	63.5	59.5	56.5
9	58.4	58.5	67.6	62.9	64.5	67.2	63.5	65.4	62.0	58.2

tective action of sulfur dioxide, which supplies an oxygen-free medium in the system. In the light of these results there is little likelihood in the idea [4] of a specific protective effect of sulfur dioxide by its chemical reaction with aldehydes.

The reason for the lower protective action of sulfur dioxide as compared to the other gases (in hydrolysis with 10% hydrochloric acid sulfur dioxide usually lacks protective action, Fig. 2) is evidently to be sought in the fact that sulfur dioxide, as Pedersen and Baker showed [4], can serve under these conditions as a source of oxygen with partial reduction to sulfur.

In hydrolysis by 10% hydrochloric acid the protective action of CO_2 is less strongly shown than in hydrolysis by sulfuric acid. This applies equally to the effects of the other gases (Figs. 1 and 2). In this connection there are interesting results [8] which show that in the hydrolysis of proteins with strong acids, cystine has a destructive action on tryptophan, and this effect is more pronounced in the hydrolysis of the proteins with 6-7 N HCl than with 6 N H_2SO_4.

The results of the experiments show that the destruction of tryptophan during hydrolysis (Figs. 1, 2) is more intense than is the increase in amino nitrogen (Table).

Thus, 3 hours after the beginning of hydrolysis by sulfuric acid in an atmosphere of CO_2 the extent of hydrolysis is 54% and the retention of tryptophan is 90%; when hydrolysis is continued for another 6 hours the increase in amino nitrogen is only 4.5% while the loss of tryptophan reaches 20%. Hence, it follows that in order to attain a given amount of hydrolysis with maximum tryptophan retention we must shorten the period of hydrolysis by increasing the corresponding acid concentration.

SUMMARY

We have studied the retention of tryptophan of fibrin when the latter is hydrolyzed by 20% sulfuric and 10% hydrochloric acids in atmospheres of nitrogen, hydrogen, carbon dioxide, and sulfur dioxide. We have shown that hydrolysis of the protein by strong acids in an atmosphere of any of these gases favors retention of tryptophan, but they show their protective actions in differing degrees. Depending on this, the effects of the gases can be arranged in the following order of decreasing action: $CO_2 > H_2 > N_2 > SO_2$. The protective action of these gases results from the oxygen-free medium which they supply, and it is shown better in hydrolysis by sulfuric acid than by hydrochloric acid. We have found that during hydrolysis the destruction of tryptophan occurs more intensely than the corresponding increase in amino nitrogen; therefore, to obtain the greatest possible hydrolysis with maximum preservation of tryptophan we must decrease the time of the hydrolysis by increasing the acid concentration.

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ANTIBODY SYNTHESIS AND THE RATIO OF PROTEIN FRACTIONS IN BLOOD SERUM UNDER THE INFLUENCE OF IONIZING RADIATION

L. G. Prokopenko

Department of Biochemistry, Kursk State Medical Institute

The study of immunogenesis in radiation sickness has become important in recent years. Interest in this question is aroused because ionizing radiation, which destroys the natural protective mechanisms, may permit the development of infectious diseases which are often disastrous to the irradiated animal [1-3]. Therefore, the study of the mechanism of inhibition of antibody production after irradiation becomes very valuable.

The opinion exists that the synthesis of antibodies and some protein fractions of the blood occurs in the same cells of the reticuloendothelial system [4]. A number of investigators consider the formation of antibodies as a modification of the synthesis of blood γ-globulin in the presence of the antigen, since the antibody and the γ-globulin are very similar in chemical and physicochemical properties such as the order of arrangement of amino acids, electrophoretic mobility, isoelectric point, etc. [5, 6].

Immunization of animals by different antigens causes proliferation of the cells of the reticuloendothelial system and transformation of the lymphocytes into plasmatic cells; a number of authors consider this of major significance in the synthesis of specific and nonspecific blood globulins [7]. This results from the repeatedly observed parallel increase in amount of globulins and titer of antibodies in immune animals [8, 9].

The effect of penetrating radiation on the ratio of protein fractions of blood serum has not been sufficiently studied. The literature contains only a few, contradictory results on this question [10-12]. We have not found any work on the study of the combined action on antibody production and production of different protein fractions by two factors so antagonistic in antibody synthesis as immunization and penetrating radiation.

We therefore feel that it is not without interest to compare the dynamics of change in the synthesis of different antibodies and the ratio of protein fractions of blood serum.

METHODS

The work was carried out on 108 rabbits given one irradiation with X-rays in a dose of 600 and 1000 r (corresponding to a sublethal dose and a dose with 50% mortality).

Irradiation was carried out on an RUM-3 X-ray apparatus at 180 kv, current strength 10 ma, skin focus distance 40 cm, filter 0.5 mm copper, output 30 r/min, without a tube.

As antigens we used a heated vaccine of Bact. parathyphi B and human blood serum. Immunization began on the second day after irradiation and was carried out by three subcutaneous injections given three times at 10-day intervals in doses of 500 million, 1 billion, and 1 billion microbes; 0.5 ml, 1 ml, and 1 ml of blood serum. The choice of two antigens very dissimilar to each other in chemical properties as indicated by the literature data [13], assures the different responses of agglutination and precipitation with these antigens.

Total blood proteins were determined by refractometer. The rabbit serum was submitted to paper electrophoresis in a veronal-medinal buffer (pH 8.6, ionic strength $\mu = 0.1$) for 3 hours at a potential gradient of 16 v/cm and a current strength of 6 ma/cm (modification of Ravich-Shcherbo and Annenkov [14]). The electrophoretogram was developed with bromophenol blue, the color was eluted with 0.01 N NaOH, and the

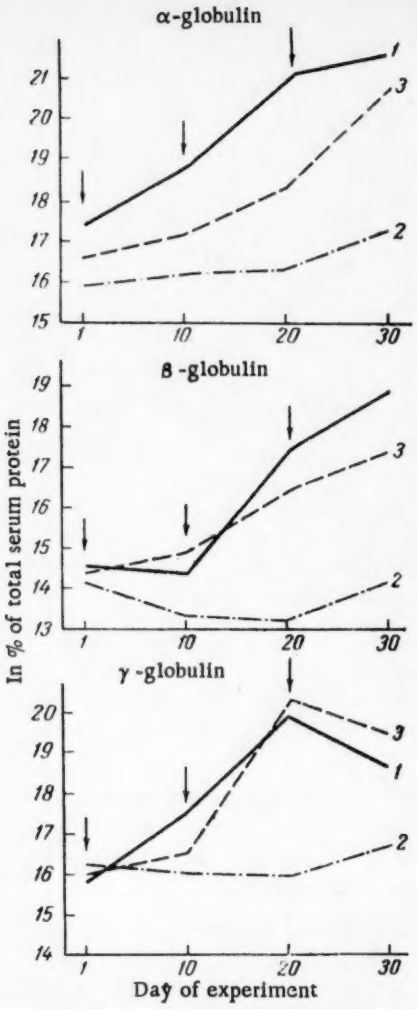

Fig. 1. Effect of irradiation (600 r) and immunization on the fractional composition of globulins. 1) immunization; 2) irradiation; 3) immunization and irradiation. Arrow shows the day of immunization.

optical density of the solution was determined on the FÉK M-1 photoelectric colorimeter. The results were treated statistically. The amount of antibody was determined by the serological reacions of agglutination and precipitation.

The quantity of leucocytes in the peripheral blood was counted in all the rabbits before irradiation and twice after it (on the 5th and 15th day). In each series two rabbits were subjected to a pathological examination.

RESULTS

Series 1 – Irradiation with a dose of 600 r. The investigation was carried out on 50 rabbits, divided into 4 groups (Table 1). As controls in the experiments with groups 2, 3 and 4 we used immunized but unirradiated rabbits. The results of the investigation, obtained by the use of each of the antigens and their complexes, showed a general similarity between them, so that we could consider them together, except when differences occurred.

In the group of irradiated but not immunized rabbits we did not find any change in the ratio of the protein fractions of the blood. In irradiated and immunized rabbits the ratio of the protein fractions was similar to that in the control rabbits which had only been immunized. The concentration of α-, β-, and γ-globulins increased on the average by 32, 17 and 29%, respectively, and the greatest increase in these fractions was found, in most rabbits, after the first immunization (Fig. 1). It should be noted that in the third group of rabbits the increase of α- and β-globulin fractions was not as clear as in the animals of the first and fourth groups. The content of albumin after the first immunization under these experimental conditions was almost unchanged, but later a fall was observed. This decrease was evidently connected with the effect of immunization; it was not found in the animals which had only been irradiated (Table 2).

The results of the experiment showed that the albumin-synthesizing function of the liver is essentially unchanged by sublethal doses of penetrating radiation. The only exception was one rabbit (out of 32 which were irradiated), in which the amount of albumin even after 10 days of the experiment fell from 3.60 to 2.69%, that is, by 25.3%. The stimulating action of immunization on the globulin-synthesizing function of the cells when they were irradiated with 600 r appeared in full measure, as the above curves demonstrate.

The titer of the agglutinins to Bact. parathyphi B in the irradiated animals after the first immunization averaged five times below the controls, although the immunological reorganization in answer to the introduction of the antigen was still considerable. This was indicated by the intensity of increase of agglutinins after the second and third immunizations. The production of precipitins in the injured rabbits was sharply damaged in the whole observation period (at the height of antibody formation, after the third immunization, the titer on the experimental animals was 1/200 and in the controls 1/6400, Fig. 2).

TABLE 1

Division of Animals of Series 1 into Separate Groups

Group	Treatment	No. of animals	No. of controls in this number
1	Irradiated	6	—
2	Irradiated + immunized by vaccine	12	4
3	Irradiated + immunized by serum	12	4
4	Irradiated + immunized by serum and vaccine	20	8

TABLE 2

Effect of Irradiation with 600 r and Immunization on the Concentration of Albumin and the A/G Coefficient* of Blood Serum

Time of analysis	Immunization		Immunization + irradiation		Irradiation	
	albumin,g%	A/G	albumin,g%	A/G	albumin,g%	A/G
Initial data	3,313±0,05	1,15±0,02	3,539±0,1	1,07±0,019	3,66±0,11	1,10±0,013
After 10 days	3,55±0,078	1,25±0,022	3,133±0,03	1,10±0,023	3,36±0,077	1,15±0,017
After 20 days	3,383±0,03	0,98±0,063	3,137±0,092	1,90±0,04	3,83±0,053	1,35±0,011
After 30 days	2,60±0,027	0,80±0,07	2,804±0,047	0,80±0,028	3,50±0,06	1,05±0,008

*A/G – albumin-globulin coefficient

TABLE 3

Division of Animals of Series 2 in Separate Groups

Group	Treatment	Total no. of animals	No. of controls in this number
1	Irradiated (1000 r)	8	—
2	Irradiated + immunization by vaccine	10	4
3	Irradiated + immunization by serum	10	4
4	Irradiated + immunization by vaccine and serum	30	12

We believe that when human serum is injected subcutaneously into irradiated rabbits, as distinct from the controls, there is no change in immunological reactivity of the organism and that the slight increase in the titer of precipitins which is found after the second and third immunization depends only on the increased dose of antigen.

Pathological study of the organs of four rabbits destroyed on the 5-12 day after irradiation showed considerable degenerative necrotic changes in the reticuloendothelia of the spleen, appendix, and mesentary lymph nodes. In the liver we observed edema of the parenchymal organs and local hemorrhages; in one rabbit there was breakdown of the liver structures.

Series 2 – Irradiation with a dose of 1000 r. The experiments were carried out with 58 rabbits. The division of the animals into separate groups is shown in Table 3.

TABLE 4

Effect of Irradiation in a Dose of 1000 r on the Concentration of Albumin and the A/G Coefficient of Blood Serum

Time of analysis	Immunization		Immunization + irradiation		Irradiation	
	albumin, g%	A/G	albumin, g%	A/G	albumin, g%	A/G
Initial data	3,30±0,04	1,02±0,013	3,48±0,055	1,10±0,02	3,13±0,041	1,15±0,04
After 10 days	3,53±0,07	1,12±0,04	3,50±0,05	1,10±0,06	2,13±0,064	0,93±0,033
After 20 days	3,37±0,069	1,05±0,03	2,43±0,023	0,91±0,015	2,18±0,038	0,93±0,05
After 30 days	2,90±0,03	1,00±0,03	2,49±0,02	0,90±0,016	2,26±0,016	0,86±0,03

Irradiation at this dose caused a considerable change in the fractional composition of the proteins of blood serum, while the character of the antigen under these conditions showed no particular effect. In the first ten days, the absolute and relative (to total serum protein) amount of γ-globulin fell (on the average before irradiation, 18.8; after irradiation, 14.9%).

After the second and third immunizations the concentration of this fraction returned to the starting level and even surpassed it somewhat, although it did not reach the level of the %-globulin in the control animals. In the irradiated but nonimmunized rabbits, the amount of %-globulin fraction during the entire period was at a low figure.

The content of β-globulins increased in all the rabbits on the average by 10-12%. The absolute quantity of α-globulins both in the controls and in the injured rabbits, increased about to the same extent, by 30-35%; the greatest increase was observed in the irradiated rabbits at the moment of their destruction. The content of this protein fraction with respect to the total amount of serum protein fell in the control animals (from 19.2 to 16.8%); in the injured animals, on the contrary, there was even a slight increase, especially after the first immunization (Fig. 3).

Determination of the amount of the α-globulin fraction in radiation sickness is evidently of considerable value, since some authors [12] have estimated by its increased concentration the response reaction of the irradiated organism to the occurrence of tissue toxins.

In the experiments of this series we also found in some rabbits a destruction of the albumin-synthesizing function of the liver, as indicated by decrease of albumin and the A/G coefficient in them (Table 4).

The dynamics of precipitin synthesis during the whole immunization period were basically the same as when the dose of 600 r was used. The titer in the irradiated animals was 40-50 times less then in the control animals. Destruction of the agglutinin formation was observed only with the first immunization. The second injection of the antigen caused a considerable rise in agglutinin titer in all the animals; in 13 of the 24 irradiated rabbits it equalled or even surpassed the controls' titer (on the average, 1/380 in the control and 1/820 in the experiments). After the third immunization the amount of agglutinins in both groups leveled off (Fig. 4).

The pathological study of the rabbit organs from animals destroyed on different days after irradiation (7-29 day of the experiment) showed the complete absence of lymphoid tissue in the spleen and spreading of the degenerative necrotic changes in the mesentary lymph nodes and intestine. Hyperplastic processes were found in the liver.

DISCUSSION

Our investigation shows that ionizing radiation affects the formation of different antibodies and nonspecific blood serum globulins unequally. The synthesis of precipitins is destroyed to a greater extent than the production of agglutinins with separate or combined immunization by the two antigens. The data which we

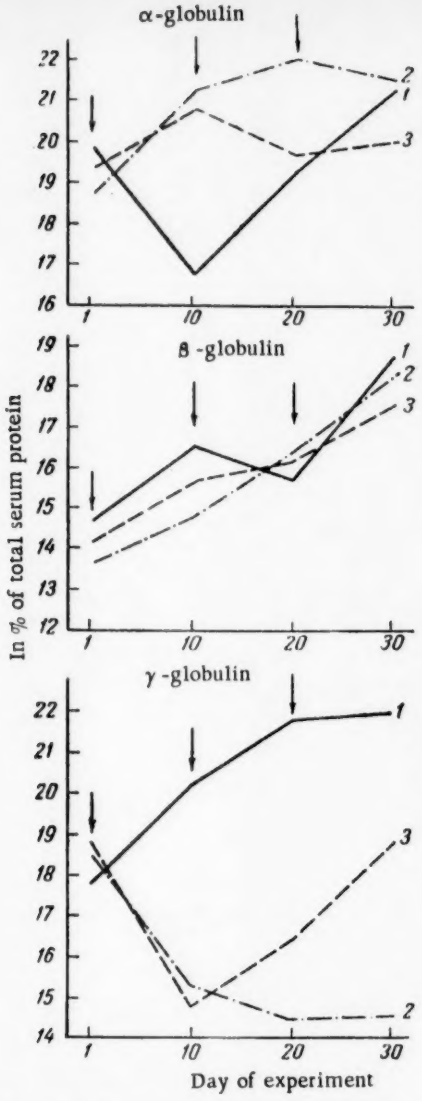

Fig. 3. Effect of irradiation (1000 r) and immunization on the fractional composition of globulins. Designations the same as in Fig. 1.

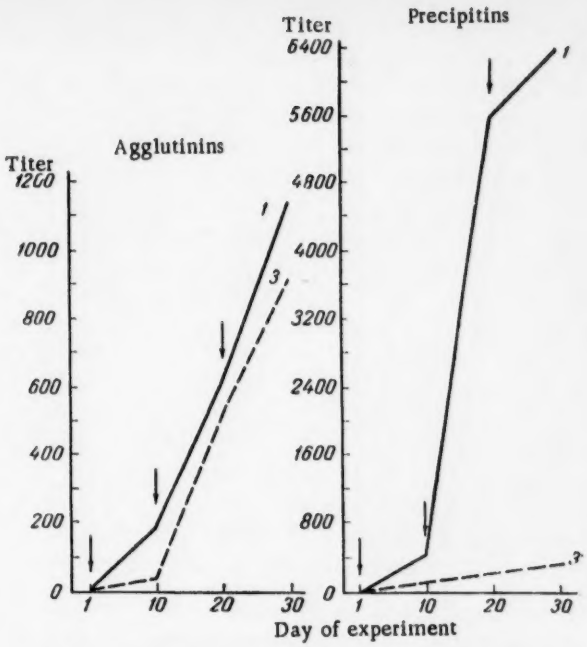

Fig. 2. Effect of irradiation (600 r) on antibody production. Designations the same as in Fig. 1.

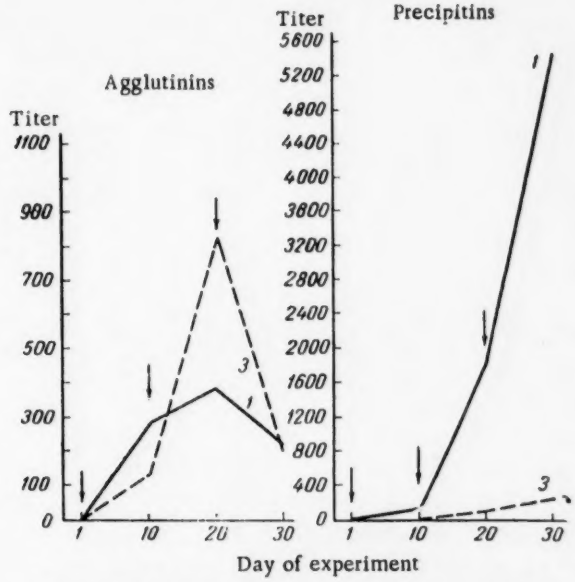

Fig. 4. Effect of irradiation (1000 r) on production of antibodies. Designations the same as in Fig. 1.

obtained can be explained by different locations or different mechanisms (different enzyme systems) for the formation of antibodies in response to the introduction of different antigens.

The literature cites the physiological lack of equivalence of different parts of the reticuloendothelial system; for example, it has been shown by labeled atoms that there is preferential formation of albumin, α-, and β-globulin in the liver, and of γ-globulin outside this organ [15-17]. Our laboratory showed that when

rabbits are poisoned by hepatotropic poisons (CCl_4 and P) there is inhibition of synthesis of the agglutinins for Bact. paracoli and Bact. parathyphi B, and the production of precipitins for human blood serum and nonspecific γ-globulin under these conditions is not destroyed. The opposite is found in poisoning by mercuric chloride which preferentially damages the kidney and intestines [13]. Of interest is the work of a number of investigators who have demonstrated the unequal participation of different parts of the reticuloendothelial system in immunogenesis in various infectious diseases [18].

It is known that under the action of penetrating radiation the earliest and most obvious changes are found in organs which are rich in lymphoid elements (bone marrow, spleen, lymph nodes, appendix [19]). As our results show, the morphological changes of reticuloendothelial cells of the liver (Kupffer cells) are less clear than those in the above organs; the globulin-synthesizingfunction of the liver is not destroyed, as is indicated by some increase in the serum of the amount of α- and β-globulin fractions. When we consider all this, we are inclined to believe that the very strong inhibition of production of precipitins as compared to agglutinins when radiation energy acts on the organism depends on the different radioresistance of areas of preferential synthesis of these types of antibodies. In our study (first series, radiation with a dose of 600 r) we found no inhibition of production of nonspecific γ-globulins, but considerable destruction of antibody synthesis.

According to the theory of Breinl and Haurowitz [4, 5] the first phase of synthesis of antibiotics and nonspecific γ-globulins consists in the formation of monomolecular polypeptide films created by the same enzyme systems. The antigens participate only in the second phase, the phase of rolling up the polypeptide films into globules and are thus templates for the antibody molecules.

When we treat our results on the basis of this theory we must admit that with irradiation by sublethal doses of X-rays, there occurs in the cells which form proteins conditions under which the effect of determinant groups of the antigen on the synthesis of polypeptide chains should be considerably weakened (perhaps because of the appearance of highly polar ions H^+, OH^-, H_3O^+, etc). However, the period of existence of these ions falls within the limits of 10^{-6} to 10^{-7} seconds [20]; hence, under the conditions of our experiments, they have disappeared long before immunization and cannot have an effect on the process of antibody formation.

Against the theory of Breinl and Haurowitz, we have the data of Gurvich [21, 22] who in a quantitative comparison of the production of two different antibodies and nonspecific γ-globulins in healthy rabbits showed the independence of production of each.

The results of our experiments agree more with the view developed by Bernet and Fenner [23] who consider that antigens do not act on the configuration of protein molecules being synthesized, but change the enzymes which occur in the process of synthesis.

The enzyme systemswhich are newly formed in the presence of antigens evidently are less stable and therefore are easily injured by toxins which occur in blood and tissues of irradiated animals; those enzymes which take part in the synthesis of nonspecific proteins in the cells are more stable to the action of penetrating radiation [24] and evidently, therefore, the process of production of nonspecific γ-globulin is suppressed only by the use of a lethal dose of X-rays (1000 r). The considerable rise in the titer of agglutinins found in our experiments after the second immunization of rabbits irradiated with 1000 r can be explained by the results in the literature on the increased permeability of the capillary walls [25, 26] and the sharp increase of virulency of the normal intestinal flora in irradiated organisms [27]. Bact. paracoli, penetrating from the intestine into the blood, can cause a further formation of antibody agglutinins. In order to confirm this idea, we carried out a special investigation on irradiated (at a dose of 1000 r) but not immunized rabbits. Under these conditions, after 15-20 days from the beginning of the experiment, the negative reaction of agglutination became positive at serum dilutions of 1/60-1/80.

However, this paper does not exclude the possibility of other mechanisms of temporary activation of agglutinin synthesis. In particular, if we hold the point of view of the possibility of participation of the Kupffer cells of the liver in the synthesis of agglutinins, then the considerable rise in their titer in our experiments can be explained also by activation of the function of these cells. Such a possibility is indicated by the proliferation of the reticuloendothelial apparatus of this organ and the accumulation in it of ribonucleic acid [28].

SUMMARY

Irradiation of rabbits by X-rays in doses of 600 and 1000 r 24 hours before the beginning of immunization sharply suppresses the synthesis of precipitins for human blood serum. The production of agglutinins for Bact. parathyphi B is decreased after the first injection of antigen and reaches the level of the control experiments or

even surpasses it after further immunizations.

In sublethal doses of X-rays there is no marked effect on the ratio of the blood protein fractions, but at doses 50% lethal (1000 r) there is a decrease in content of albumin and γ-globulin and some rise in the content of α- and β-globulins.

In conclusion we express thanks to Professor M. I. Ravich-Shcherbo for suggesting the theme and directing the work.

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SOME MATERIALS ON THE DYNAMICS, LOCALIZATION, AND METABOLISM OF PEPTIDES IN PLANT LEAVES

Zh. A. Medvedev and Shen Chiang-hsia

Department of Agricultural and Biological Chemistry, K. A. Timiryazev Agricultural Academy, Moscow

Peptide compounds of amino acids are usually present in plant tissues in larger or smaller amounts [1-6]. Except for some peptides with specific structures (of the type of glutathione), this fraction consists of a variety of compounds which are intermediates in the splitting or synthesis of proteins. The composition and functional role of plant peptides have been very little studied, and only in recent years have these compounds attracted any attention. The interest in the peptide fraction of plant and animal tissue is not a matter of chance; it is connected with the great progress which has been made in a comparatively short space of time in the study of the biosynthesis of proteins.

Surveys of studies on the composition and biological role of the peptides of microorganisms, animal, and plant tissues have recently been made [7, 8] and therefore we will discuss here only some of the methods of this problem.

It has been shown at the present time that protein synthesis occurs in several stages and that in the final stage the synthesis of specific proteins is determined by ribonucleic acid of the cellular organoids, which serves as the matrix for this synthesis. However, this final stage of the synthesis is preceded by specific activations of the amino acid which occur under the influence of complex enzyme systems and low molecular weight, soluble RNA, localized in the plasma fluid of the cells, or more correctly, in the supernatant liquid separated from the microsomes by prolonged centrifuging at 105,000 g [9, 10]. The presence of this preliminary activation of the amino acids has also been observed in plants whose activating enzyme complex is very similar in composition and nature of action to that of animal tissues [11-15]. In many communications on the study of amino acid activation it has been shown that one of the steps in this activation is adsorption by the activated amino acid fraction of cytoplasmic RNA which reacts with the activating enzyme systems. Then all the remaining reactions, still very little studied, use these amino acids in the synthesis of proteins without their entering a completely free state. When labeled amino acids are introduced into the cell, they clearly show rapid absorption of soluble RNA and transfer of the labeled atoms into the microsome, where they are already found in the composition of the proteins bound to the highly polymerized RNA of these organoids.

In the surveys mentioned above [7, 8] many facts were given which indicated the possible role of peptides as intermediate products in protein synthesis. This makes it apparent that if the peptides are actually one of the stages in this process, then their formation should be an intermediate step between activation of the amino acids in the plasma and the final protein synthesis which occurs on some organoid, though this does not exclude the possibility that the intermediate peptide may remain bound to some RNA fraction. A number of investigators have favored this view by showing that RNA isolated from samples with different origins holds just the peptide products especially firmly [16-19].

In previous work on peptide metabolism in plants which was carried out in the Department of Agricultural and Biological Chemistry of the Timiryazev Agricultural Academy [20-21], we have already touched on some questions of peptide participation in protein synthesis. The peptides used in these experiments were labeled

with C^{14} and showed the possibility of their direct uptake in synthetic processes. We also observed in plant tissues mixtures of peptides which were not separated from RNA when purified, and the increased intensity of protein synthesis was accompanied by simultaneous increase of these mixtures [19]. However, these facts still do not give sufficient assurance that these effects actually are produced by fragments of normal protein synthesis.

We have already mentioned above that different phases in protein synthesis occur in different cell fractions. Therefore, if peptides are actually intermediate products of this synthesis, then their first formation should be connected with some definite cell fraction. Most probably this fraction is the plasma fluid, since it is there that the primary activation of the amino acids occurs and the RNA phosphorus metabolism, which is related to protein synthesis, is found most intensely [22]. It is also interesting to determine the rate of peptide uptake in protein synthesis under conditions of different intensities of this synthesis. Both these problems are involved in the present study.

METHODS

Most of the experiments were carried out with beans (Phaseolus vulgaris) raised in a greenhouse in large Mitscherlich vessels on a sand culture with rich Hellriegel mixture. We usually used month-old plants for the experiments. In two experiments on study of the dynamics of growth of amino acids and peptides in different fractions we used leaves of the bean Termopsis officinalis.

In the first series of experiments with beans we studied the changes in content of amino acids and peptides in different intracellular fractions of leaves of different ages under conditions in which the rate of protein synthesis was varied experimentally by supplying a nitrogen deficit or by nitrogen feeding. The nitrogen deficit was not established at the beginning of the development of the plant, but after a month of normal growth, so that there was no difference in initial growth and development of the plants. Nitrogen starvation was produced in this case by washing the vessel with a large volume of tap water and distilled water, followed by addition to the vessel of all the required ingredients except nitrogen. After 5-6 days growth on such a medium, the plants showed obvious signs of nitrogen starvation (paling of the leaves). Nitrogen feeding to strengthen the synthesis of amino acids and proteins was begun in most cases after a five to seven-day period of nitrogen starvation. We usually used $Ca(NO_3)_2$ for feeding, adding an amount equivalent to its content in the Hellriegel mixture.

Fractionation of the Material. For analysis, the plant leaves were fractionated into vacuole fluid, plasma fluid, and structures which remained after removal of the liquid fractions. In some experiments the plastids were isolated separately. Isolation of the vacuole and plasma fluids was carried out by compression according to the method of Mason and Phillis [23] with some variations [24, 26].

The plasma fluid obtained by this process contains different proteases in an active state, as shown by the rapid splitting of proteins in it as a result of autolysis (Table 2).

In Table 1, as an example, we give the results of some determinations of the quantitative ratios of these fractions calculated on wet weight of the plants. We determined these ratios in all our experiments and for all samples, since they were needed for further calculations.

Isolation of the plastids from the leaf homogenates was carried out by centrifugating at 2000 g after preliminary removal of the nucleic and undestroyed cells. The leaf samples for analysis varied from 50 to 150 g. Two samples were used for the isolation. In the latter part of the work each sample was used for two analyses; thus, the greater part of our numerical material is an average of four analytical determinations.

Determination of Amino Acids and Peptides. Amino nitrogen was determined in the plasma fluid and the different extracts by the manometric method of Van Slyke after precipitation of the protein with 5% trichloracetic acid, using not less than 24 hours exposure of the solution to this acid in the refrigerator (for full precipitation of the protein). The peptide content was estimated by the increased content of amino nitrogen in the deproteinated extracts after their complete hydrolysis by 20% HCl. The difference in content of amino nitrogen before and after hydrolysis was due to the nitrogen bound as peptides. In most of the experiments carried out in 1958, deproteination was run by two parallel methods: precipitation by TCA and dialysis through cellophane. We used dialysis because we thought that the large peptides, according to evidence in the literature,

TABLE 1

Quantitative Ratios of Isolated Fractions, (in percent of wet weight of plants)

Object of analysis	Vacuole fluid	Plasma fluid	Water bound in the structure	Dry residue
Leaves, 2–3 layers	22.6	52	14.3	11.0
" 4–5 "	24.9	46	15.8	13.2
" 6–7 "	17.3	54	14.0	14.3

TABLE 2

Splitting of Proteins of Different Cellular Fractions from Beans in Autolysis, (in mg protein in the sample)

Period of autolysis	Plasma fluid protein			Plastid protein			Cytoplasm proteins (without plastids)		
	experiment number								
	1	2	3	1	2	3	1	2	3
Initial sample	35	51	41	66	91	226	163	132	290
24 hour autolysis	11	18	27	54	64	196	101	67	141
100 hour autolysis	8	8	16.5	48	61	151	65	38	136

TABLE 3

Content of Different Types of Nitrogen-Containing Substances in the Cellular Fractions of Bean Leaves Depending on Conditions of Supplying Nitrogen, (in mg N per 100 g wet weight of leaves)

Object analyzed	Plasma fluid			Residual structures		
	free amino nitrogen	peptide bound amino nitrogen	protein nitrogen	free amino nitrogen	peptide bound amino nitrogen	protein nitrogen
Protein removed by TCA						
Leaves of layers 2-3						
Control	18.6	7.9	9.7	30.0	58.8	442
7 days after nitrogen starvation	14.4	5.9	11.6	19.1	42.0	240
4 days after feeding $Ca(NO_3)_2$	34.9	10.6	12.6	43.3	42.3	352
Leaves of layers 4-5						
Control	19.0	10.9	17.8	34.6	54.2	533
7 days after nitrogen starvation	15.9	14.1	8.7	26.2	45.2	315
4 days after feeding $Ca(NO_3)_2$	49.7	23.4	18.7	47.5	40.7	372
10 days after feeding $Ca(NO_3)_2$	16.8	7.1	12.7	43.4	58.3	455
Protein removed by TCA						
Leaves of layers 2-3						
Control	25.4	0.4	9.3	26.6	18.0	328
4 days after feeding $Ca(NO_3)_2$	27.0	7.9	11.3	25.2	17.5	346
Leaves of layers 4-5						
Control	17.5	0.7	9.5	18.6	26.4	370
4 days after feeding	29.6	12.9	24.2	26.0	23.7	579
Protein removed by dialysis						
Leaves of layers 3-5						
Control	23.8	0.4	—	21.7	24.4	
4 days after feeding	34.2	9.0	—	28	16.7	

TABLE 4

Content of Amino and Protein Nitrogen in Plastids from Bean Leaves Depending on Conditions of Nitrogen Supply

Characteristics of object analyzed	Content of protein nitrogen in the resulting form	Free amino nitrogen	Peptide bound amino nitrogen	Nonprotein nitrogen in % of protein
Leaves of plants with insufficient nitrogen	64.4	5.9	0	9,2
Leaves of plants after feeding $Ca(NO_3)_2$	47.0	6.8	0	14 5

TABLE 5

Content of Different Types of Nitrogenous Substances in Leaves of the Perennial Bean Termopsis officinalis of Different Ages (in mg per 100 g wet weight of leaves)

Leaf fraction	Yound spring leaves			Peptide bound amino nitrogen		
	free amino nitrogen	peptide bound amino nitrogen	protein nitrogen	free amino nitrogen	peptide bound amino nitrogen	protein nitrogen
Vacuole fluid	5.8	0.8	—	6.3	0	—
Plasma fluid	45.8	0	19.9	35.5	0	10.8
Residual structures	36.8	15.0	681	37.4	12.1	352.5

could be precipitated by TCA along with the proteins [3]. However, in our experiments, precipitation of the proteins with TCA and their separation by dialysis gave very similar results when the amino acids and peptides were determined in the filtrate and by evaporation of the dialyzate.

Experiments with Introduction into the Plants of Peptides Labeled with C^{14}. Peptides totally labeled with C^{14} were obtained by pepsin and trypsin hydrolysis of proteins labeled on the carbon. Such proteins were obtained from leaves of beans kept for four days in a chamber which contained $C^{14}O_2$. At the same time, we prepared a complete hydrolyzate of these proteins. The pepsin hydrolyzate of the proteins consisted, on the average, of peptides with the dimensions of 5.5 amino acid residues, the trypsin, of 2.3 amino acid residues. Hydrolyzates were produced under identical conditions as to activity, pH [6, 8], salt concentration, etc. All the details of setting up the experiments to obtain peptides labeled on the carbon have been described in our previous papers [20, 21]. Some special points in carrying out the experiments in the present work are indicated below.

RESULTS

In Table 2, we give data which show the comparative rate of autolysis of proteins from different cellular fractions. The relatively rapid splitting of the plasma-fluid proteins is clearly evident.

Reaction mixture: plasma fluid or suspension of organoid in isotonic solution of KCl + phosphate buffer, pH = 6.0. Incubation carried out at 37° in the presence of traces of thymol (antiseptic).

The results on change in content of proteins, amino acids, and peptides in different leaf fractions of bean and Termopsis depending on conditions of nutrition and age are given in Tables 3-5. It follows from a comparison of these data that in the fraction of "free amino nitrogen" occur both free amino acids and free amino groups of peptides. The fraction of "peptide-bound amino nitrogen" corresponds only to those amino groups which are liberated after hydrolysis. If we assume conditionally, for instance, that all the peptides in the plant are tetrapeptides, then the amount in them would be 25% more amino nitrogen than we determined in the "bound" form.

TABLE 6

The Utilization of Different Hydrolyzates, Labeled on the Carbon of the Proteins, for Protein Synthesis and Respiration when they are Introduced in to Bean Leaves (activity of added hydrolyzates equal to 22,000 counts/min per ml; nitrogen content in them 0.7 mg/ml)

Experiment No.	Nature of leaves used for experiment	total activity in 10 g wet weight, counts/min	Total leaf protein				Carbon dioxide bound as $BaCO_3$			
			weight in mg per 10 g wet weight	activity in counts/min per 10 mg	total activity of protein per 10 g wet wt. in counts/min	activity of protein in % of total	wt. $BaCO_3$ in mg per 10 g wet weight	activity in counts/min per 10 mg	total $BaCO_3$ activity per 10 g wet wt.	activity of $BaCO_3$ in % of total
			Entire hydrolyzate							
1	Control									
	Old Leaves, layers 2-3	20406	270	204	5521	27.0	259	178	4618	22.6
	Young leaves, layers 4-5	47830	248	470	11685	24.4	508	142	7217	15.1
	After nitrogen starvation									
	Old leaves, layers 2-3	15335	276	108	2983	19.4	249	154	3842	25.0
	Young leaves, layers 4-5	27031	180	204	3678	13.6	387	136	5262	19.5
			Trypsin Hydrolyzate							
	Control									
	Old leaves, layers 2-3	18150	331	177	5856	32.2	251	71	1787	9.8
	Young leaves, layers 4-5	26720	247	244	6034	22.6	383	64	2455	9.2
	After nitrogen starvation									
	Old leaves, layers 2-3	15608	377	95	3582	22.9	249	94	2348	15.0
	Young leaves, layers 4-5	23758	295	102	3012	12.6	361	63	2276	9.6
			Entire hydrolyzate							
2	Old leaves	7962	197	69	1360	17.1	158	133	2112	26.5
	Mature leaves	23731	261	234	6107	25.7	269	147	3966	16.7
			Pepsin hydrolyzate							
	Old leaves	13595	180	135	2430	17.8	179	88	1579	11.8
	Mature leaves	23625	258	209	5404	22.9	300	93	2796	11.6

The results summarized in the tables show a sufficiently clear picture of the synthesis of peptides after supplementary addition of nitrogen fertilizers which also stimulate the synthesis of amino acids and proteins. Increase in the synthesis of amino acids and proteins does not have any particular interest in the present case. Here we devote ourselves only to the particularly sharp jump in accumulation of amino acids in the plasma fluid. In our experiments, there was also a 24-hour sample, but no sharp change could be observed in the first 24 hours. A still sharper increase in amino nitrogen in the plasma fluid after feeding the plant with $Ca(NO_3)_2$ was previously observed in the corn plant [24], and from this we can assume that the plasma fluid is the chief point of accumulation of amino acids when they are synthesized in excess. The data relating to the peptides has greater interest in the present case since the change in their content in the different cell fractions has not previously been studied. Here we can note two circumstances. First, under the ordinary conditions of plant development, peptides are present especially in the residual structures (not in the plastids, but evidently in the microsomes). In the plasma fluid there is little, or none at all (in Termopsis). However, after feeding the plant the peptide content is greatly increased, almost entirely on account of increase of the content in the plasma fluid. We should also remark that in these analyses we did not specially determine those peptide fractions which were firmly bound to RNA, since TCA precipitation when it is long in contact with the cell fractions separates this fraction to a considerable extent from RNA. We can assume that after activation of the amino acids in the

plasma fluid there is also partial peptide synthesis, which peptides are then transferred to the organoids for further "building up". The synthesis of peptides on the surface of further-phosphorylated RNA has recently been shown in vitro [25]. It is certain that our materials are insufficient for exact conclusions as to the localization of peptide synthesis, since for this we would need experiments with shorter exposure; this is hard to get when inorganic compounds are introduced through the roots, since they require preliminary reduction by the nitroreductase system. However, we believe that these materials clearly show, first, that peptides in plants are formed not only by splitting of proteins, but also when their synthesis is stimulated, and, second, the main points of localization of the peptides are the cellular structures (although there is the possibility of their synthesis in the plasma fluid).

We showed earlier the probable use of the peptides by the plants for the synthesis of proteins [20, 21].

In the present work, we have set ourselves the problem of continuing these studies in two directions. We consider it interesting, first, to compare the use of peptides and amino acids for protein synthesis under conditions of different rates of such synthesis (depending on age and conditions of nitrogen supply), and, second, to compare with each other, two groups of enzyme hydrolyzates: the pepsin, which consists of large peptides, and trypsin, whose products are the result of deeper splitting of the proteins. The hydrolyzates, equalized in all physicochemical and chemical properties except degree of splitting the protein, were introduced into the plant through the petiole. Some of the leaves [15-17], placed in a test tube with the petioles in the hydrolyzates, were put into a glass chamber in the dark; all the carbon dioxide given off by the leaves was absorbed in alkali and precipitated as $BaCO_3$. We determined the radioactivity of the protein and nonprotein fractions of the leaves separated from the petioles. The total exposure was 3 hours. In Table 6 we give the chief results of this work.

From the data of this table, we see most clearly the ratio of amount of C^{14} included in the protein and given off as carbon dioxide in respiration. In all cases of the use of partial enzyme hydrolyzates, this ratio was strongly shifted to the side of the proteins, which is evidence for their partial utilization in synthesis without preliminary splitting. The amount of hydrolyzates entering through the petioles varied widely but it was always greater in younger leaves. The specific activity of the proteins was also higher in young leaves but it was impossible to judge from this activity alone the rate of their synthesis, since the rate of C^{14} uptake in the proteins depends on many factors and most of all on the rate of dilution of the labeled components by the unlabeled ones contained in the cell. We did not determine the degree of this dilution. Preliminary starvation of the plants did not increase, but decreased their ability for synthesis and metabolism in the first hours after beginning the experiment.

However, it is characteristic that if the specific activity of the proteins in younger leaves is always 1.5-2 times as high as in old leaves, which may be connected with the more active absorption of the hydrolyzate, yet the specific activity of $BaCO_3$ in young leaves is always lower than in old, and in mature leaves approaches the specific activity of old leaves. At the same time, the rate of oxidative processes in young organs is higher. Such behavior of young leaves is apparently related to the fact that the ratio of amount of amino acids oxidized to amount of other products oxidized in them is lower than in old leaves. It seems to us that this behavior with age may be very characteristic for the study of age changes of nitrogen and carbon metabolism in plants.

In many cases, the nonspecific strong adsorption of amino acids and peptides, can affect the results of the determination of protein activity; this effect can be avoided by running special control experiments. For this we added to a homogenate of young leaves a definite amount of labeled amino acids and peptides which corresponded in activity to the amount which entered the leaves in 3 hours, and also 0.01% dinitrophenol to inhibit protein synthesis. After a corresponding exposure, the proteins were isolated from the homogenate, carefully washed and transferred like the proteins in the main experiments. The specific activity of these proteins was 5 and 14 counts/min per 10 mg, respectively, for the complete and pepsin hydrolyzates of the protein, that is, only about 3 4% of the specific activity which was found when the hydrolyzate was introduced into the living leaf. It is therefore certain that the observed adsorption had no important effect on the results obtained in the experiments which we have described.

SUMMARY

The plasma fluid obtained from leaves after removal of the vacuole fluid and freezing is an active and easily obtained cytoplasmic fraction.

When the bean plant is fed with nitrogen fertilizers there is a considerable synthesis of amino acids and peptides in the leaves and the content of this group of compounds increases chiefly in the plasma fluid. In the control plants (before feeding),the amino acids, and especially the peptides, are localized chiefly in the residual intracellular structures.

When proteins, partly hydrolyzed enzymatically and labeled with C^{14}, are introduced into the leaves,there is a direct uptake of peptides in the synthesis of proteins corresponding to the rate of this synthesis in the organs studied.

These results indicate intermediate formation of peptides in the synthesis of proteins.

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THE BIOSYNTHESIS OF VALINE AND ISOLEUCINE IN THE RIPENING WHEAT EAR

V. L. Kretovich and Z. S. Kagan

A. N. Bakh Institute of Biochemistry, Academy of Sciences, USSR, Moscow

The mechanism of biosynthesis of essential amino acids with branched chains,(valine, leucine, and isoleucine)nas attracted much attention by investigators in recent times.

The study of the biosynthesis of valine, leucine, and isoleucine, aside from its own interest for an understanding of the mechanisms of synthesis of these essential amino acids, is also interesting because the biosynthesis of valine is important as a precursor in the biosynthesis of coenzyme A [1] and pantothenic acid [2], and the biosynthesis of isoleucine,as a precursor in thebiosynthesis of carotene [3] and isoprene derivatives [4].

However, the majority of investigators on the mechanism of the biosynthesis of valine, leucine, and isoleucine have worked with microorganisms (bacteria, yeasts, and other lower fungi). The question of the biosynthesis of these amino acids in higher green plants has not been studied at all. It is only known that homogenates of sprouts of different plants are able to catalyze the transamination of α-ketoglutaric acid and a number of amino acids, including valine, leucine, and isoleucine [5]. The reverse of this transamination reaction has not been shown in plants. It is also known that transamination of α-ketoisovaleric acid (the ketoanalog of valine) and α-keto-β-methyl-n-valeric acid (the ketoanalog of isoleucine) with glutamic acid is followed in the bacterium E. coli [6] and the fungus N. crassa [6, 7] by the stage of biosynthesis of valine and isoleucine. In addition, it is known that in some plants, especially in the leaves and bulbs of the tulip, the ketoanalogs of valine and leucine are present [8].

The purpose of the present work is to investigate the synthesis of valine and isoleucine and their closest nitrogen-free precursors, the corresponding ketoacids, in the ripening wheat ear.

METHODS

Chemical Synthesis of α-Ketoisovaleric and α-Keto-β-methyl-n-valeric Acids

α-Ketoisovaleric acid (the ketoanalog of valine) was synthesized by the method of Perkin and Simonsen [9, 10]. The principle of the synthesis is the condensation of acetone with hippuric acid in the presence of acetic anhydride and sodium acetate with the formation of the azlactone of N-benzoyl-α-amino-β, β-dimethylacrylic acid, followed by hydrolysis of the azlactone to the free ketoacid.

A mixture of 100 g of hippuric acid, 250 g acetone, 100 g sodium acetate, and 190 g acetic anhydride (all reagents first purified and dried) was heated on a boiling water bath for 6 hours. After it had cooled, the orange liquid was poured into a large amount of water. The orange precipitate of azlactone was washed with water, a dilute solution of Na_2CO_3, once more with water, and was recrystallized from aqueous ethanol. We obtained almost colorless crystals, m. p. 98-99° (according to the literature 98-99° [9]; 99-100° [10]; 100.2-101.2° [11]). Some quantity of the azlactone could be obtained further from the alcoholic mother liquors by diluting the solution with water and filtering the precipitate. The yield of azlactone was 34.5 g. Thirty-two g of this azlactone was hydrolyzed in 180 ml of concentrated HCl on a water bath for 6 hours. After the mixture had been cooled, the precipitate was filtered and the orange filtrate was repeatedly extracted with ether. The

Content of Amino Acids in Wheat Ears in μmoles per g of Dry Weight

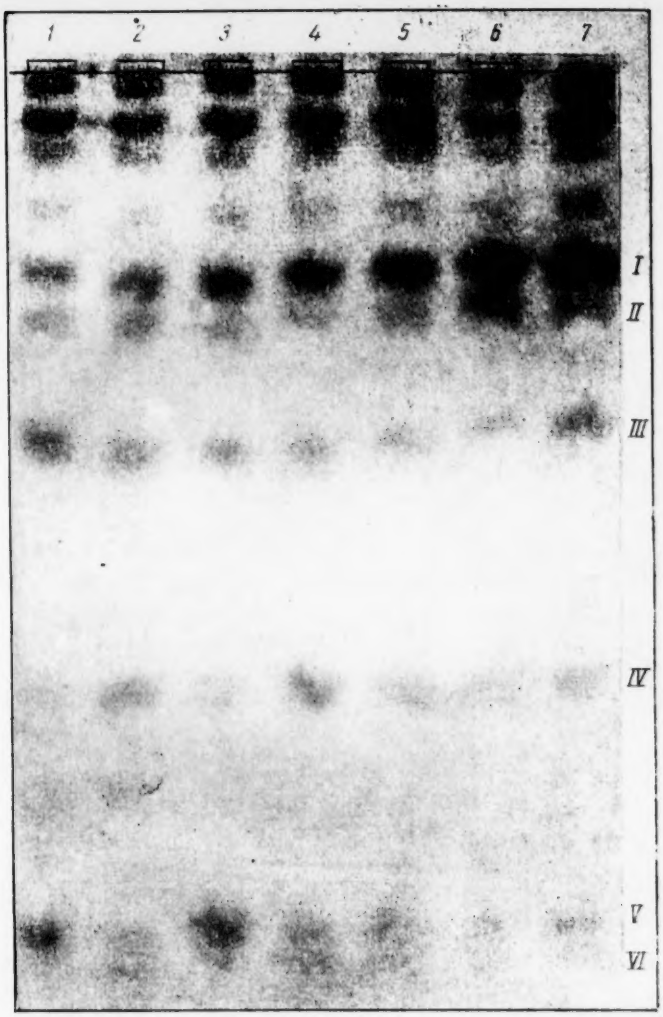
Amino acids	Variant of experiment				
	water	ketoanalog of valine	ketoanalog of isoleucine	ketoanalog of valine + $(NH_4)_2HPO_4$	ketoanalog of isoleucine + $(NH_4)_2HPO_4$
Experiment of July 15, 1958. Exposure 10 hours (moisture of material 59.4%)					
Alanine	3,88	3,46	3,33	3,46	3,74
Aspartic acid	1,57	1,24	1,52	1,52	1,42
Glutamic acid	2,01	1,26	1,12	1,54	1,49
Serine	2,74	1,48	2,82	1,64	2,74
γ-Aminobutyric acid	1,91	2,20	2,16	2,20	2,08
Valine	1,37	3,22	1,32	2,76	0,99
Leucine + isoleucine	1,56	1,99	3,42	1,26	3,16
Experiment of July 18, 1958. Exposure 10 hours (moisture of materials 58.9%)					
Alanine	5,36	4,10	3,83	4,79	4,37
Aspartic acid	1,10	1,10	0,92	1,10	1,10
Glutamic acid	1,79	1,33	1,24	1,44	1,16
Serine	2,04	2,39	2,02	1,81	3,31
γ-Aminobutyric acid	1,25	1,44	1,06	2,01	1,94
Valine	0,97	3,50	0,81	2,43	0,81
Leucine + isoleucine	1,74	1,71	2,86	1,26	3,12

slightly yellow ether solution was dried over anhydrous Na_2SO_4; a residue was left after the ether was distilled off in a vacuum. α-Ketoisovaleric acid had b. p. 55° at 3 mm Hg or 65° at 11 mm (according to the literature, 65° at 11 mm Hg [10], 65-69° at 10 mm [12], 65-67° at 10 mm [13], 170-175° at normal pressure [14]). The white platelets of this amino acid had m. p. 16.5° (according to the literature, 16° [9], 31° [13]). Yield 6.3 g. To prepare the sodium salt, we dissolved the drystals in a small amount of water, neutralized with dry $NaHCO_3$, and evaporated the solution in a vacuum desiccator. The 2,4-dinitrophenylhydrazone of this α-ketoacid had m. p. 194° (according to the literature, 194° [10], 194-195° [15]).

The synthesis of α-keto-β-methyl-n-valeric acid (the ketoanalog of isoleucine) was carried out by the method suggested by one of us [16] based on the analogous condensation of hippuric acid with methylethyl ketone (310 g).

However, when we poured the orange reaction mixture into a large amount of water a crystalline precipitate of the azlactone did not separate, but instead a heavy, oily, orange liquid, which after being washed with water in a separatory funnel, then with dilute Na_2CO_3, and then again with water, was hydrolyzed directly in 150 ml of concentrated HCl for 6 hours on a boiling water bath. When acid was added to this liquid, a crystalline precipitatecame down but dissolved when the reaction mixture was heated. At the end of the hydrolysis a crystalline precipitate again appeared (evidently benzoic acid) and was filtered off; the orange liquid was repeatedly extracted with ether, the extract was dried over anhydrous Na_2SO_4 and evaporated. The residue was distilled in a vacuum. α-Keto-β-methyl-n-valeric acid had a b. p. 62-63° at 7 mm Hg or 72° at 11 mm (according to the literature, 72-74° at 11 mm [12], 73° at 10 mm [17], 70-75° C at 11 mm [18], 78-80° at 12 mm [19], 84° at 15 mm [20], 90° at 21 mm [21]). The m. p. of the white crystals was 36.5° (according to the literature, 35° [20], 34.5-35.5° [12], 30° [18-19], 38-40° for the optically active isomer of this ketoacid [17]). Yield 3.8 g. The sodium salt was prepared by the method described above. M. p. of the 2,4-dinitrophenylhydrazone of this ketoacid 168° (according to the literature, 168-169° for the racemate of this α-ketoacid [17], 176° for either of its stereoisomers [17, 22].

Of the other methods for the synthesis of α-keto-β-methyl-n-valeric acid [13, 14, 17-22] the method of Wieland is especially important because of the relative simplicity and high yield, and the method worked out by Meister [17] which consists in the oxidative deamination of D-isoleucine or L-alloisoleucine by a preparation of D-amino acid oxidase from liver or L-isoleucine or D-alloisoleucine by a preparation of L-amino acid oxidase from snake venom. In these cases the corresponding L, D-stereoisomers of this α-ketoacid are obtained in pure form.



Chromatogram of free amino acids in extract from wheat ears. Experiment of July 15, 1958. Exposure 10 hours. Paper buffered with 0.067 M phosphate buffer, pH 6.2. Solvent – o-cresol saturated with the same buffer. Experimental variants: I) ketoanalog of isoleucine + $(NH_4)_2HPO_4$; 2) ketoanalog of valine + $(NH_4)_2HPO_4$; 3) ketoanalog of isoleucine; 4) ketoanalog of valine; 5) Na_2HPO_4; 6) $(NH_4)_2HPO_4$; 7) water. Indicatedamino acids: I) alanine; II) glutamine; III) γ-aminobutyric acid; IV) valine; V) isoleucine; VI) leucine.

The resulting sodium salts of the ketoanalogs of valine and isoleucine were chromatographically pure; in paper chromatography with a solvent butanol-acetic acid-water (6:1:5) and development by an acid-alkali indicator according to Lugg and Owerell [23] they gave only one spot with an R_f value which corresponded to 0.55 and 0.68.

Enzymatic Identification of α-Ketoanalogs of Valine and Isoleucine

The ketoanalogs of valine and isoleucine were also verified by us by an enzymatic method. For this purpose we studied their ability to be transaminated with glutamic acid in a mouse-liver homogenate.

White mice were decapitated and their livers ground in the cold with 0.065 M phosphate buffer, pH 7.45. The homogenate was filtered through a double layer of gauze and used in the following variants: 1) control, without additions; 2) sodium glutamate; 3) ketoanalog of valine; 4) ketoanalog of isoleucine; 5) ketoanalog of valine + sodium glutamate; 6) ketoanalog of isoleucine + sodium glutamate. Final concentration of all compounds in the homogenate 0.05 M. After incubation for 3 hours at 37°, the samples were fixed by adding two volumes of ethanol. The amino acid content in the alcohol extract was determined by paper chromatography, using as the solvent o-cresol saturated with a phosphate buffer, pH 6.2. The chromatogram showed clearly that in the homogenate of mouse liver the ketoanalogs of valine and isoleucine actively transaminated with glutamic acid to form valine and isoleucine respectively.

Course of the Experiments

Ears of winter wheat No. 2453 grown on the fields around the K. A. Timiryazev Moscow Agricultural Academy were gathered at the stage of young maturity. The experiments were carried out on July 15 and 18, 1958. In each variant of the experiments, we used 40 ears. After the lower part of the stem was cut off under water, the bottom of the stem of the ear was put into solutions of the following compositions: 1) a solution of the ketoanalog of isoleucine + $(NH_4)_2HPO_4$; 2) a solution of the ketoanalog of valine + $(NH_4)_2HPO_4$; 3) a solution of the ketoanalog of isoleucine; 4) a solution of the ketoanalog of valine; 5) aqueous solution of Na_2HPO_4. Aqueous solution of $(NH_4)_2HPO_4$. 5. Water, control. The final concentration of all the compounds was 0.05 M. The ears were saturated with these solutions by the transpiration current for 3 and 10 hours, and then from each variant of the experiment we took 20 ears and ground and weighed the grains and scales. For each variant we took an average sample of 20 g. They were frozen in a Dewar vessel with solid CO_2. The remaining material of all 7 variants was mixed and three samples taken to determine the moisture. Then the frozen samples were ground in a mortar cooled with a mixture of alcohol and solid CO_2 and the material was fixed by immersion in 100 ml of 96% ethanol. After 1-2 days extraction, the solution was filtered and the residue treated with 70 ml of 80% ethanol; after several days the filtrate was added to the first portion of extract and the residue was carefully washed with 80% ethanol so that the final volume of extract was 200 ml. The final alcohol concentration was 80%.

Quantitative Paper Chromatography of the Amino Acids

The content of amino acids in the alcohol extracts of wheat ears was determined by quantitative paper chromatography by the method of Kretovich and Uspenskaya [24, 25].

We used Schleicher and Schull chromatographic paper No. 2040. For the determination of alanine, γ-aminobutyric acid, valine, and sum of leucine and isoleucine, the paper was buffered with 0.067 M phosphate buffer, pH 6.2, and as solvent we used o-cresol saturated with the same buffer. We used single-phase descending chromatography with two passages of solvent. For determination of aspartic and glutamic acids and serine we used paper buffered with 0.067 M NaOH-phosphate buffer, pH 12 and phenol saturated with the same buffer. For determination of alanine we placed on a 2.0-cm-long starting line 0.2 ml of alcohol extract; for determination of all the other amino acids, 0.6 ml. Three dilutions (for alanine 2.5, 5.0 and 7.5 μg, for valine, γ-aminobutyric acid and isoleucine 2.5, 5.0, and 10.0 μg, and for aspartic and glutamic acids and serine, 2.5, 7.5 and 15.0 μg were deposited on the same sheet of chromatographic paper as standards, and for each chromatogram we constructed its calibration curve. We ran not less than 3-5 parallel chromatograms and used the average values. The chromatograms were rendered visible by a 1% ninhydrin solution in acetone which contained 4% or 2% acetic acid when phenol or o-cresol were used as solvents. The five amino acids were eluted by adding 0.5 ml of 0.5% solution of $CdCl_2$ in 40% methanol with later addition of 9.5 ml of 40% methanol solution. The intensity of the color of the eluate was determined on the photoelectric colorimeter with a green filter in a cuvette with length of optical path 3.0 cm.

RESULTS AND DISCUSSION

The results of the determination of content of free amino acids in wheat ears which had absorbed different solutions by transpiration current are shown in the table and also on the photograph of the chromatogram (figure). Since the increase in valine and isoleucine when their ketoanalogs were introduced into the ear with 10-hours exposure was considerably higher than with 3 hours, we analyzed quantitatively only the material from the 10-

hour experiment; the results of the 3-hour experiments were analogous and differed only in the smaller value of synthesis of valine and isoleucine.

The table shows clearly that in the ripening wheat ear, from the ketoanalogs of valine and isoleucine the corresponding valine and isoleucine are synthesized. Although isoleucine was determined as the sum with leucine, yet on the chromatogram obtained by passing the solvent twice, it was clearly shown that just isoleucine was synthesized.

In almost all cases the synthesis of valine and isoleucine from the ketoacids was accompanied by the synthesis of γ-aminobutyric acid. The mechanism of the synthesis of this compound is not clear, but the fact once again emphasizes the great role of γ-aminobutyric acid in the amino acid metabolism of plant organisms. In all cases the synthesis of valine and isoleucine from the ketoanalogs is accompanied by a marked loss in glutamic acid and alanine and the small decrease in glutamine.

Thus, from the preceding experimental material it follows that in the ripening wheat ear, valine and isoleucine are synthesized from the corresponding ketoanalogs. In the synthesis of these amino acids the transamination reaction takes part, and the source of the amino group is primarily alanine and glutamic acid.

Our results do not rule out the possibility of direct reductive amination of the ketoanalogs of valine and isoleucine in the green plant by ammonium ion. Thus, the reductive amination of the ketoanalogs of valine and leucine occurs in the cells of Bacillus subtilis [26, 27] and by enzymatic preparations of D- and L-amino acid oxidases [28, 29]. It has also been shown that there is amination by ammonia of the ketoanalog of norvaline in the presence of crystalline L-glutamic acid dehydrogenase [30].

SUMMARY

We have described in detail a chemical method for the synthesis of the ketoanalogs of valine (α-ketoisovaleric acid) and isoleucine (α-keto-β-methyl-n-valeric acid).

We have shown that the introduction of the ketoanalogs of valine and isoleucine into wheat ears results in their transformation into valine and isoleucine. At the same time the content of glutamic acid and alanine in the ears decreases, which indicates their participation in transamination and the biosynthesis of valine and isoleucine.

The introduction into the ear of these ketoacids leads to the synthesis of γ-aminobutyric acid.

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THE DISTRIBUTION OF LIPIDS IN THE CYTOPLASM OF LIVER CELLS IN ACUTE RADIATION SICKNESS

V. D. Blokhina and N. N. Demin

For an explanation of the metabolic disturbances which occur in the living organism during the development of radiation sickness it is very interesting to study the quantitative and qualitative lipid content in various morphological components of the cells of different tissues. The role of lipids in metabolism and their significance for the properties of the cellular microstructure must be considered.

In an earlier study [1] we showed that, in acute radiation sickness in rabbits and rats, one day after X-ray irradiation there was an increase in lipid content in the mitochondria and microsomes of liver tissue cells.

For the further study of the disturbance of lipid metabolism with radiation damage, we have made a study of the quantitative relations between free and bound lipids in the whole cytoplasm and separately in the hyaloplasm of the liver cells of rabbits injured by γ-radiation. These studies also cast light on the problem of the change in state of the lipoprotein complexes in the cytoplasmic organella and in the hyaloplasm of liver cells in acute radiation sickness. The study of the degree of binding of lipids in the cell in the development of acute radiation sickness is important in view of the very great significance and varied physiological roles of cellular lipoproteins.

The determination of the content of free and bound lipids in the present work is based on their difference in extractability by organic solvents.

MATERIALS AND METHODS

The experiments were carried out on male rabbits weighing 2.5-3.5 kg. The animals before and during the experiments were kept on the ordinary animal house diet of oats and vegetables.

Acute radiation sickness was produced by a single irradiation with γ-rays from Co^{60} in a dose of 1000 r (strength of irradiation 500 r/min). Irradiation at this dose led to acute radiation sickness in the rabbits. Most of the animals died, beginning from the fifth to the seventh day after exposure.

As a rule, in each experiment we killed by air embolism two rabbits of different weights, one irradiated and one healthy control. For 16 hours before death the animals were deprived of food. The liver was removed from the dead animals and washed with 0.9% NaCl solution, then ground in a homogenizer with ten times the volume of isotonic sucrose solution. The preparation of cytoplasm and hyaloplasm of the rabbit-liver cells was carried out by the previously described process [2].

To obtain the dry weight of the preparation, we freed an amount equal to 5 ml from sucrose by dialysis in a cellophane bag against 2 liters of distilled water for 48 hours, then transferred the contents quantitatively to a container and dried it at 60° to constant weight.

Determination of the content of free lipids was carried out as follows: the preparation of cytoplasm or hyaloplasm, 10 ml, was shaken with 25 ml of ligroin for 3 min at room temperature, centrifuged at 2000 rpm for 5 min, and the upper ligroin layer was decanted into a separate flask. The operation of shaking with fresh portions of ligroin was repeated 4 times. The combined lipid extracts were evaporated at room temperature to small volume and the lipid content was determined in aliquot parts by a modified Bloor method [3].

TABLE 1

Content of Different Lipid Fractions in Whole Cytoplasm of Rabbit Liver Cells in Acute Radiation Sickness (in % of dry weight of cytoplasm)

Time of study	Lipids extracted with ligroin	Lipids extracted with methanol-chloroform mixture	Fatty acids in residue after extraction
Normal	4.4±0.8	19.5±1.8	10.3±2.5
24 hours after irradiation	5.2±0.2	31.7±4.1	15.7±3.4
72 hours after irradiation	6.5±1.1	19.9±1.2	8.0±2.3

TABLE 2

Content of Different Lipid Fractions in the Hyaloplasm of Rabbit Liver Cells in Acute Radiation Sickness (in % of dry weight of hyaloplasm)

Time of study	Lipids extracted with ligroin	Lipids extracted with methanol-chloroform mixture	Fatty acids in residue after extraction
Normal	10.8±1.5	16.8±1.6	10.6±1.8
24 hours after irradiation	11.3±1.4	20.7±2.3	18.5±4.4
72 hours after irradiation	12.1±2.2	18.8±3.0	13.7±3.0

For determination of the content of total lipids the lipids were removed from a fresh portion of 10 ml of cytoplasm or hyaloplasm by the method of Folch et al. [4]. The residue after numerous extractions with a methanol-chloroform mixture was submitted to alkaline hydrolysis with 30 times the amount of 8% alcoholic alkali for 4 hours, and then the fatty acids were removed from the hydrolyzate by the process described by Romantsev [5]. The total lipid content was found by adding the amount of lipids found by the Folch method and the fatty acids isolated by later alkaline hydrolysis.

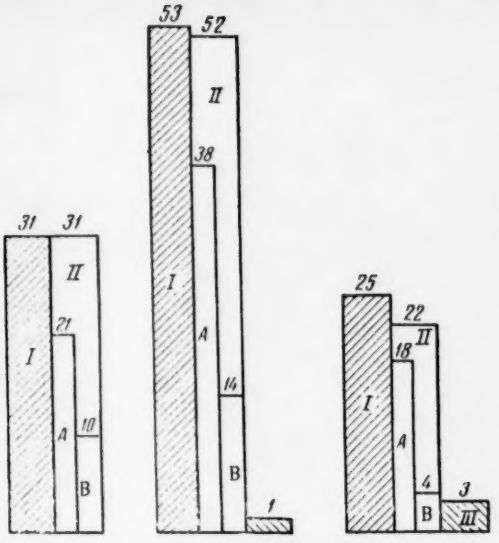
The quantity of bound lipids was determined from the difference between the content of total and free lipids. By the difference between the amount of lipids removed by the methanol-chloroform mixture and extracted by ligroin we found the amount of relatively more loosely bound lipids.

The hyaloplasm made up about 40% of the total dry weight of liver-cell cytoplasm. On the basis of this value and the results of the determination of the lipid fractions which interested us, in the whole cytoplasm and separately in the hyaloplasm, we calculated the approximate content of these fractions in the cytoplasmic microstructures (rounded off to a whole number).

RESULTS AND DISCUSSION

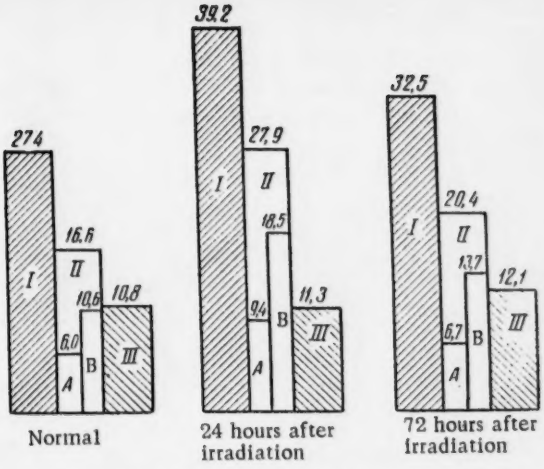
The determination of the content of the different lipid fractions in preparations of whole cytoplasm and hyaloplasm was carried out 24 hours (5 experiments) and 72 hours (4 experiments) after the irradiation injury: at the beginning and at about the highest point in the acute radiation sickness.

In Tables 1 and 2 are given the average content of lipids extracted by different organic solvents from preparations of whole cytoplasm and hyaloplasm of liver cells in normal rabbits and those with acute radiation sickness. There, we also give the average results of determinations of the content of fatty acids after alkaline hydrolysis in the residues from the same preparations of cytoplasm and hyaloplasm after extraction. On the basis of these experimental results we have calculated the content of weakly bound and firmly bound lipids and we have expressed the approximate content, in wt. %, of all the lipid fractions in



Normal | 24 hours after irradiation | 72 hours after irradiation

Fig. 1. Content of different lipid fractions in cytoplasmic organoids of rabbit-liver cells in acute radiation sickness (in % of dry weight) I) total content; II) bound lipids (A - weakly bound, B - firmly bound); III) free lipids.



Normal | 24 hours after irradiation | 72 hours after irradiation

Fig. 2. Content of different lipid fractions in hyaloplasm of rabbit-liver cells in acute radiation sickness (in % of dry weight) I) total content; II) bound lipids (A - weakly bound, B - firmly bound); III) free lipids

the cytoplasmic organoids. These results are shown graphically for the cytoplasmic organoids in Fig. 1 and for the hyaloplasm in Fig. 2. Also, in Table 3, we give the numerical data which characterize the dynamics of quantitative and qualitative change in lipid composition of the cytoplasmic organoids and hyaloplasm in percent of the normal and the ratio between the separate fractions in percent.

According to our data, rabbit-liver tissues in the normal animal are very rich in lipids. Their total content is about the same in the cytoplasmic organoids and in the hyaloplasm, and equals about 30% of the dry weight. This indicates an important role for lipids in this organ.

At the same time, the ratios between the different fractions of the lipids of the cytoplasmic organoids and the hyaloplasm of liver cells are very different from each other. In the first, practically all the lipids occur in the bound state, preferably in the weakly bound form (about 70%); there are only traces of free lipids here. Evidently, the lipids of the cytoplasmic organoids occur as lipoproteins which have great significance for the structure and function of the morphological components of cytoplasm. In the hyaloplasm, there is another distribution – about 40% of the lipids are free, and the bound lipids belong chiefly to the firmly bound fraction (40% against about 20% weakly bound).

In the early period after irradiation (after 24 hours), corresponding to the results of the previous study [1], we observed a rise in total lipid content in the hyaloplasm and especially in the cytoplasmic organoids, corresponding to about 40% and about 70% of the normal.

In the hyaloplasm this increase occurred because of a rise mostly in the content of the bound lipids, which were especially hard to extract; thereupon, the amount of free lipids fell markedly (from about 40 to about 30% of the total lipid content).

At this time, there occurred in the cytoplasmic organoids a still more marked increase in content of bound lipids, but the accumulation of weakly bound lipid compounds prevailed in them (this fraction increased by 80%). We should say that in the cytoplasmic organoids there could be observed a small, in absolute amount, but relatively substantial, increase in quantity of free lipids, reaching a value of about 10 times greater than the normal.

Seventy-two hours after irradiation, at the highest point of the acute radiation sickness, we found in the experimental rabbits further changes in amount and distribution of the lipid fractions in the cytoplasmic com-

TABLE 3

Quantitative and Quaitative Changes in Composition of Lipids of Cytoplasmic Organoids and Hyaloplasm in Rabbit-Liver Cells in Acute Radiation Sickness

Time of study	Total fat content, % of normal	Free		Weakly bound		Firmly bound	
		in % of normal	in % of total content	in % of normal	in % of total content	in % of normal	in % of total content
Cytoplasmic Organoids							
Normal	—	—	0.3	—	68	—	32
24 hours after irradiation	+70	(10 times more than normal)	2	+80	72	+40	26
72 hours after irradiation	—20	(30 times more than normal)	12	—15	72	—60	16
Hyaloplasm							
Normal	—	—	39	—	22	—	39
24 hours after irradiation	+43	+5	29	+57	24	+75	47
72 hours after irradiation	+15	+12	37	+12	21	+29	42

ponents of the liver cells. The total lipid content fell in this period. In the hyaloplasm, it still remained above normal (by 15%), but in the cytoplasmic organoids it decreased below normal (by about 20%).

In the hyaloplasm this decrease in total lipids depended on the considerable lessening in the amount of the bound fraction; the content of free lipids increased. The ratio between the different lipid fractions of hyaloplasm thus came closer to the normal.

In the cytoplasmic organoids at the same period in the development of acute radiation sickness, there was further considerable damage to lipid metabolism. The content of bound lipids fell below normal; the decrease in amount of firmly bound lipids fell especially sharply, reaching about 60% of the normal; the content of weakly bound lipids was about 15% less than normal. At the same time, the content of free lipids in the cytoplasmic organoids was raised, reaching about 30 times normal. This gave a sharp change in composition of the lipids of the cytoplasmic organoids.

We can conclude from our data that with the development of acute radiation sickness, there occurs in rabbit-liver cells not only a change in the total lipid content in the cytoplasmic components, but also a considerable redistribution of the lipid fractions, chiefly in the cytoplasmic organoids.

When radiation sickness developed under our experimental conditions, we found in the hyaloplasm basically only a quantitative change in lipids; the ratio between their separate fractions was changed slightly, but only after a definite period (24 hours) from application of radiation (a small decrease in amount of free lipids and some increase in the firmly bound fraction).

In the case of the cytoplasmic organoids, we noted an increased concentration (almost doubled) of weakly bound lipids 24 hours after irradiation, a sharp and lasting absolute and relative increase in amount of free lipids (this was about 0.3% of the total lipid content in the normals, 2% after 24 hours, and 12% after 72 hours from the irradiation), and a decreased amount of the firmly bound fraction (from about 32% in the intact rabbit to 16% after 72 hours from irradiation). It is interesting that 24 hours after irradiation we observed some increase in amount of firmly bound lipids in the cytoplasmic organoids (by about 40% above normal), but this did not lead to an increased relative content of this fraction. When the radiation sickness developed further, both the absolute and relative amounts of firmly bound lipids decreased sharply and were below the starting level which was characteristic for healthy animals. At the same time, the relative content of weakly bound lipids of the cytoplasmic organoids remained practically unchanged in spite of the above-noted considerable rise in the amount of this fraction 24 hours after irradiation, and, perhaps, there was some fall below normal after 72 hours.

It may still be that in the development of radiation sickness there is at first a slow splitting of lipoproteins in the cytoplasmic organoids and in the hyaloplasm of rabbit-liver cells, which then becomes stronger in the cytoplasmic organoids especially for the most stable complexes) with decreased ability of these microstructures to bind free lipids. In the hyaloplasm, on the other hand, the firmly bound lipids are the most stable.

The data agree well with the previous investigations [6] in which it was shown that in rabbits 1-3 days after injury by a lethal dose of X-rays, the content of the lipoprotein fraction decreased in the mitochondria of liver cells.

SUMMARY

In intact rabbits, the lipids make up about 30% of the dry weight of the cytoplasmic organoids and hyaloplasm of the liver cells; in the cytoplasmic organoids the weakly bound lipids make up about 70% and the firmly bound lipids about 30% of the total lipid content; there are only traces of free lipids. In the hyaloplasm weakly bound lipids comprise about 20%, firmly bound lipids 40%, and free lipids 40%.

During the development of acute radiation sickness, there is a temporary increase in lipid content in the hyaloplasm, chiefly from the bound lipids (predominantly the firmly bound fraction).

In the cytoplasmic organoids 24 hours after irradiation there is a considerable accumulation of bound lipids (especially the weakly bound fraction), and a marked increased in content of free lipids; the relative content of the different fractions of bound lipids probably does not change. After 72 hours there is a sharp decrease in amount of bound lipids (especially the firmly bound fraction) and a marked further rise in content of free lipids. This leads to considerable disturbance both in the amounts of lipid fractions of the cytoplasmic organoids and the ratio between them.

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AMINO ACID METABOLISM IN HOMOGENATES OF MUSCULAR AND NERVOUS TISSUE IN CONNECTION WITH ADENYLIC ACID REAMINATION

P. A. Kometiani

Department of Biochemistry, The Institute of Physiology of the Georgian SSR Academy of Sciences, Tbilisi.

It is well known that nervous and muscular activity are accompanied by the release of ammonia. One of the main sources of ammonia in nervous and muscular tissue is the cyclic reaction of deamination of the adenylic system [1, 2, 3]. It has been demonstrated in the laboratory that inosinic acid reamination may be accomplished at the expense of the amino groups of glutamate, aspartate and γ-aminobutyrate and of β-alanine in nervous tissue, and at the expense of aspartate and β-alanine in muscle.

Klein [4], who investigated the metabolism, in brain homogenates, of all the amino acids which participated in the resynthesis of the adenylic system, found that they underwent all the known reactions of amino acids namely, deamination, deamidation, decarboxylation and transamination. These various reactions were found to be closely interconnected. Any change in the concentration of any of the amino acids caused changes in the concentrations of the remaining components. Thus, when brain homogenates were incubated with glutamate, there was an increase not only in the content of its immediate decarboxylation product – γ-aminobutyrate, but also in aspartate and β-alanine. The introduction of either aspartate or of γ-aminobutyrate caused an increase in the other amino acids. It is also noteworthy that the addition of inosinate caused a decrease in all of the amino acids mentioned. This pointed to the possible existence of a cyclic mechanism for the deamination of adenylate and its derivatives. In such a system inosinate would be expected to behave as a coenzyme in the deamination of amino acids.

The interconvertibility of the amino acids in this system makes it impossible to recognize directly the primary source of ammonia. The present work deals with the various pathways of amino acid turnover and with the conditions optimal for the amination of inosinate.

METHODS

Rat brain and muscle were used throughout.

Preparation of homogenates. Brain homogenates were prepared in the cold from pooled brains of two rats. The homogenates were diluted sixfold with a saline solution containing 0.04 M potassium phosphate buffer pH 7.2 and 0.06 M potassium chloride. Two ml of the homogenate (300 mg of tissue) was used in each experiment. The composition of the reaction mixture (final concentrations) was as follows: potassium chloride, 0.06 M.; potassium phosphate buffer, 0.04 M; sodium fluoride, 0.012 M; manganese chloride, 0.003 M; to this were added, depending on experimental conditions: amino acids, 0.007 M, inosinic acid, 0.0033 M, adenosinetriphosphate, 0.006 M and pyridoxal phosphate, 0.0005 M. The final volume of the reaction mixture was 3 ml. The muscle homogenates were prepared in the same reaction mixture, but were diluted 2.5-fold. Dialyzed homogenates were employed in a number of cases. These were dialyzed four hours against the saline solution (0.04 M phosphate buffer and 0.06 M potassium chloride).

TABLE 1

Changes in Amino Acid Content of a Brain Homogenate During Incubation at 37° for 1 Hour (Amino acids in mmoles/100 g wet tissue)

Amino acids	Before incubation	After incubation	
		without ATP and PALPO	with ATP and PALPO
Aspartic acid	0.26	0.29	0.20
Glutamic acid	0.88	1.07	0.82
Asparagine + glutamine	0.40	0.06	0.26
γ-aminobutyric acid	0.25	0.30	0.18

TABLE 2

Changes in Amino Acid Content During the Incubation of Brain Homogenates in the Presence of Glutamate and Coenzymes (Amino acids in mmoles/100 g wet tissue)

Amino acids	Before incubation	After incubation	
		with glutamate	with glutamate and coenzymes
Aspartate	0.24	0.29	0.23
Asparagine + glutamine	0.38	0.12	0.25
γ-aminobutyrate	0.27	0.35	0.28

Amino acid determination. The homogenates were incubated one hour and treated with four volumes of ethanol. The mixture was filtered and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in a measured volume of water. The amino acids in the solution were determined quantitatively by a paper-electrophoretic method according to Grassman et al. [5], with the following modification. An aliquot of the extract, corresponding to 30-40mg of brain, or to 40-60 mg of muscle, was applied to the center of a paper strip, 4 x 35 cm. Electrophoresis was carried out for 4-5 hours under a potential difference of 400 V, with a current 2-3 ma, the solvent used being pyridine-acetic acid buffer (0.05 M acetic acid, 0.01 M pyridine, pH 4.0).

Dicarboxylic amino acids were separated under these conditions from their decarboxylation products. Glutamine and asparagine remained together with the neutral amino acids. The determination of the sum of asparagine and glutamine was carried out after a secondary electrophoretic run at pH 1.9 (0.1 M formic acid + 0.06% NaCl).

The quantitative determination of amino acids was carried out according to Dent [6]. The bands were developed by treatment with 1% ninhydrin in butanol and were eluted from the paper by shaking for one hour with 2 ml of 20% methanol. The color was stabilized by the addition of 1 ml of 0.5% cadmium nitrate. In a number of experiments the amino acids were determined according to Rosen [7], but glycol was used instead of the isopropyl alcohol.

RESULTS AND DISCUSSION

There is a body of published data on the content and turnover of free amino acids in brain and muscle. The modern methods of chromatography and electrophoresis on paper have greatly improved the accuracy of

TABLE 3

Changes in Amino Acid Distribution During Incubation of Muscle Homogenates for 1 Hour at 37° (Amino acids in mmoles/100 g wet tissue)

Amino acids	Before incubation	After incubation	
		without additions	with added coenzymes
Aspartate	0.05	0.06	0.03
Glutamate	0.35	0.38	0.30
Asparagine + glutamine	0.25	0.12	0.18
β-alanine	0.02	0.03	0.01

available information, and also permitted discovery a number of new facts. Ansell and Richter [8] published detailed information on the distribution of the amino nitrogen of the free amino acids in the brain. The striking feature in brain is its high content of glutamate, aspartate, their respective amides and of γ-aminobutyrate. It was also found [9] that the free amino acid content of brain increased post mortem as a result of proteolytic activity. This was further confirmed by Weil-Malherbe and Green [10]. Once the participation of glutamate in the elimination of ammonia was established [11], further work continued on the content of glutamate and glutamine in brain in various functional states, and also on the role of these compounds in ammonia production [12-15].

The free amino acid content of nervous tissue was also investigated in the present work, where of primary interest was the effect of incubation conditions on the content of certain amino acids (Table 1).

A consideration of the results shown in Table 1 reveals that during incubation (without additions) there occurred, on one hand, an increase in the decarboxylic amino acids (aspartate and glutamate) and in γ-aminobutyrate and, on the other hand, a marked decrease in the content of the amides (glutamine and asparagine). The divergence of the present results from some published data [8, 10] is probably due to the fact that the homogenates in the present work were prepared with phosphate buffer, and phosphate is known to activate glutaminase. It must also be borne in mind that the increase in dicarboxylic amino acid content of the "metabolic pool" would also lead to an increase in all the remaining amino acids (with the exception of the amides). It is therefore clear that the increase in free amino acids observed during the incubation of brain homogenates was due not only to the action of proteases, but also to the interconversions within the free amino acid pool and to the hydrolysis of amides.

The addition of coenzymes – adenosinetriphosphate (ATP) and pyridoxal phosphate (PALPO) – altered considerably the distribution of amino acids during the incubation of the homogenates (Table 1). It appears that where the brain homogenates were incubated with ATP and PALPO, glutamine and asparagine hydrolysis was inhibited, and there was a considerable loss of glutamate, aspartate and γ-aminobutyrate, probably due to transamination, oxidative deamination and the cyclic deamination of adenylate. There was therefore a decreased content of the above-mentioned amino acids when ATP and PALPO were added to the incubation mixture. At the same time a sparing effect on the amides was observed.

The formation and removal of γ-aminobutyrate was demonstrated in the experiments where the homogenate was incubated with added glutamate (Table 2). When ATP and PALPO were added together with glutamate to the incubation mixture, the γ-aminobutyrate level at the end of the incubation was found to be lower than when glutamate alone was the supplement. This suggests that the rate of the cyclic deamination of the adenylic system predominated over the rate of glutamate decarboxylation.

It will also be seen from Table 2 that, during the incubation of the homogenates in the presence of ATP and PALPO, there was also a slight decrease in aspartate content, apart from the decrease of γ-aminobutyrate discussed above. This change would be the net sum of two processes: de novo synthesis of aspartate and its loss.

TABLE 4

Amino Acid Levels in Muscle Homogenates After Incubation in the Presence of Aspartate and Coenzymes (Amino acids in mmoles/100 g wet tissue)

Amino acids	Incubation	
	with aspartate alone	with aspartate and coenzymes
Glutamate	0.38	0.28
Asparagine + glutamine	0.08	0.12
β-alanine	0.016	0.012

In the presence of ATP the formation of aspartate from asparagine is not likely. However, aspartate can also be formed by transamination from glutamate and oxaloacetate. Loss of aspartate could take place along a number of pathways, such as asparagine synthesis, decarboxylation and transamination to inosinate. The latter arises through the stepwise dephosphorylation and deamination of the adenylic system.

When the homogenates were incubated in the presence of added aspartate, the further addition of ATP and PALPO stimulated the formation of asparagine and β-alanine. There was a concomitant fall in the levels of all other amino acids with the exception of the two amides (glutamine and asparagine).

The experiments with aspartate supplementation resembled those with added glutamate in that in

TABLE 5

Ammonia Production During Incubation of Brain and Muscle Homogenates with the Dicarboxylic Amino Acids, Inosinate and Coenzymes. Control: homogenate, homogenate + ATP, homogenate + inosinate (Ammonia in mmoles/100 g wet tissue)

Brain homogenate			
glutamate	ATP + PALPO + glutamate	inosinate	inosinate + PALPO + glutamate
0.02	0.28	0.12	0.23
Muscle homogenate			
muscle homogenate	ATP + PALPO + aspartate	inosinate	PALPO + inosinate + aspartate
0.05	0.32	0.15	0.28

brain homogenates supplemented with aspartate, and in the presence of ATP and PALPO, amide hydrolysis was inhibited and the reactions of transamination and decarboxylation were stimulated.

Results of similar experiments with muscle homogenates were somewhat different from those obtained with brain. While glutamate was the more intensively metabolized amino acid in brain, this position in muscle was occupied by aspartate. We have demonstrated earlier [3] that muscle homogenates could decarboxylate aspartate, but not glutamate. It was further established that aspartate and β-alanine, but not glutamate or γ-aminobutyrate, were utilized in muscle for the reamination of inosinate. There were also differences in the total levels of free amino acid amino nitrogen, which was found to be about 45 mg% in brain and 25 mg% in muscle. This difference was mainly due to the high levels of glutamate and γ-aminobutyrate in brain. Unlike brain homogenates, muscle homogenates on incubation showed only a slight increase in the free amino acid level, which was probably due to the low level of proteolytic activity.

Experiments on the change in amino acid distribution on incubation of muscle homogenates (Table 3) showed that, if no ATP was added, then at the end of a one-hour incubation period there was, as in the case of brain, an increase in dicarboxylic acids and β-alanine, and a considerable decrease in the level of the two amides.

TABLE 6

Ammonia Production During the Incubation of Muscle Homogenates in the Presence of Either Aspartate, Asparagine of β -alanine. Control: Homogenate + ATP + PALPO (Ammonia in mmoles/100 g wet tissue)

Aspartate	Asparagine	β -alanine
0.37	0.13	0.08
0.28	0.14	0.06
0.39	0.20	0.10
0.32	0.23	0.07

If ATP and PALPO were added to the muscle homogenates, the main effect was one of sparing the amides, which remained on comparatively high levels. There was a concomitant fall in the levels of the dicarboxylic amino acids and of β -alanine (Table 3).

ATP addition to muscle homogenates affected particularly the aspartate levels.

On the basis of published work, and also from our earlier observations [3], it would appear that aspartate metabolism took place mainly along three pathways: decarboxylation, amidation and transamination. In this multiplicity of metabolic pathways, aspartate was unique among the free amino acids of muscle.

When aspartate was added to muscle homogenates, further supplementation with ATP and PALPO caused an increase in amide content and a relatively marked fall in β -alanine level. Due to the inhibition of amide hydrolysis, the final level of glutamate at the end of the incubation was lower in the presence of ATP and PALPO than when these coenzymes were absent (Table 4). It should be noted that we have previously reported the finding that muscle glutamate did not participate in inosinate reamination [3].

Thus, these data confirm the conclusions drawn by Klein from the study of changes in amino acid distribution on incubation of brain homogenates [4]. The changes in amino acid distribution are also due to the ease with which they interact with each other. In the present work the addition of ATP was found to inhibit amide hydrolysis, which caused a final lower level not only of the dicarboxylic amino acids, but also of β -alanine and γ -aminobutyrate. The expenditure of amino acids in the reversal of adenylate deamination must be suspected as the prime cause of the decrease in amino acid levels on addition of ATP to the homogenates. This may be demonstrated by the stimulating effect of inosinate on this phenomenon and the simultaneously increased ammonia production. This is illustrated in Table 5, in which are presented the data for ammonia production during the incubation of brain homogenate in the presence of glutamate and of muscle homogenate in the presence of aspartate.

It follows from Table 5 that inosinate in the presence of PALPO stimulated ammonia production. As mentioned above, this increase in ammonia production was accompanied by a fall in the levels of the dicarboxylic amino acids and of their decarboxylation products. Ammonia production in the presence of inosinate should be primarily related to the cyclic deamination of the adenylic system, while in the presence of ATP the participation of glutamine and asparagine in the production of ammonia should not be overlooked.

It has been established [16] that ATP acts as a coenzyme in the synthesis of glutamine from glutamate and ammonia. When the homogenates were incubated in the presence of ATP the levels of the amides remained high due to the shift of the reaction towards amide synthesis. At the same time ATP is converted, by a stepwise dephosphorylation and deamination, to inosinate. The latter compound acts as an acceptor of amino groups in the reamination to adenylate, and thus stimulates the utilization of amino acids. When the homogenate is incubated with inosinate, amide neosynthesis does not take place and ammonia production is due to the cyclic deamination of adenylate.

When the homogenates were incubated with ATP and the amides, ammonia production was lower than in the case of supplementation with the dicarboxylic amino acids (Table 6).

It follows from Table 6 that, if ammonia production be taken as a criterium, aspartate was a better donor of amino groups in muscle than was either asparagine or β -alanine. Asparagine possibly reacts directly with inosinate after deamination or, in analogy with the reaction with keto-acids, discovered by Meister [17], participates directly in transamination. The latter alternative seems unlikely, since it would be expected to produce twice the amount of ammonia which would arise from an equivalent amount of aspartate.

It should be noted that the values quoted in Table 6 were obtained using a centrifuged muscle homogenate,

TABLE 7

Ammonia Production During the Incubation of Brain and Muscle Homogenates in the Presence of ATP, Glutamine and Asparagine (Ammonia and Substrates in mmoles)

Additions	Brain		Muscle	
	ammonia	decomposition of added substrate, %	ammonia	decomposition of added substrate, %
Asparagine, 7 mmoles	3.4	49	2.6	37
Glutamine, 7 mmoles	5.1	71	3.7	53
Asparagine, + ATP	1.0	14	0.7	10
Glutamine + ATP	1.8	26	1.2	17
ATP, 6 mmoles	2.7	45	5.1	7.3

TABLE 8

The Conversion of Aspartate to β-alanine during the Incubation of Muscle Homogenates, and the Effect of Inosinate on this Process. Control: Dialysed homogenate + ATP (β-Alanine in mmoles/100 g wet tissue)

Aspartate + PALPO	Aspartate + inosinate	Aspartate + PALPO + inosinate
0.06	0.02	0.01
0.10	0.03	0.03
0.08	0.02	0.01
0.06	0.04	0.02

containing only the soluble adenylate deaminase. It was demonstrated by Muntz [18, 19] that the soluble deaminase was specifically activated by ATP. Increase in ammonia production in brain homogenates on the addition of ATP and glutamate was reported previously from this laboratory [2]. This stimulation did not take place when glutamate was substituted by γ-aminobutyrate and glutamine. In brain and muscle respectively glutamate and aspartate, in the presence of ATP, stimulated ammonia production to a much larger degree than did their respective deamination or decarboxylation products. The intensification of ammonia production by ATP may be explained by its activating effect on the adenylate deaminase.

The failure of the amides of the dicarboxylic amino acids to participate directly in the cyclic deamination of adenylate was demonstrated in experiments where the rates of ammonia production in homogenates were measured during incubation in the presence of ATP with the addition of the amides (cf. Table 7).

When a brain homogenate was incubated 1 hour in the presence of ATP and glutamine, the ammonia produced amounted only to 26% of that theoretically available through the complete deamination of the glutamine added. In a similar experiment with a muscle homogenate, in the presence of ATP, only 17% of the added glutamine was deaminated. When asparagine was added to the two tissue homogenates, 14% was deaminated in brain homogenates and 10% in muscle. On the addition of ATP alone to brain homogenate, 45% of the amino nitrogen present was liberated in the form of ammonia in 1 hour. In the case of muscle this value reached 73% in the same time. If the homogenates were incubated in the presence of asparagine or glutamine, but without added ATP, ammonia production was considerably increased, but in this case this was due to amide hydrolysis.

It follows from Table 7 that, firstly, ATP inhibited the decomposition of the amides and, secondly, that the rate of adenylate deamination exceeded that of amide decomposition in both brain and muscle.

The final experiment dealt with the formation of β-alanine from aspartate in muscle homogenates.

Aspartate decarboxylation in brain was first reported by Awapara et al. [20] and, simultaneously, by Roberts and Frankel [21]. The formation of β-alanine from aspartate was also mentioned by Klein [4]. The presence of this reaction in muscle is demonstrated by the results in Table 8.

It will be seen from the data presented in Table 8 that β-alanine formation from aspartate was stimulated by the addition of pyridoxal phosphate. If inosinate was added as well, β-alanine levels fell. This fall may be explained by the intensification of amino acid utilization in the reamination of inosinate to adenylate.

Following the communications from this laboratory [1, 2, 3] on the pathways of adenylate transamination in muscle, there appeared reports on the presence of this reaction in bone tissue and in certain bacterial preparations. Abrams and Bentley [22] showed that a soluble enzyme from bone marrow could synthetize adenine and guanosine nucleotides, respectively, from the corresponding monophosphates of inosine and xanthosine. It was found that the amino group for inosinemonophosphate was provided by aspartate, while xanthosinemonophosphate amination required glutamate. The authors suggested that inosinate condensed with aspartate with the formation of adenylosuccinate. The formation of this intermediate, catalyzed by enzymatic preparations obtained from dried yeast, was demonstrated by Carter and Cohen [23].

Lieberman [24] studied the mechanisms of nucleotide synthesis and found that inosinate was activated by means of a new co-factor. This co-factor, which brought about the phosphorylation of inosinate, was shown to be guanosinetriphosphate. It was established that ATP also took part in the reamination of inosinate. The authors believe that the tole of ATP was confined to the rephosphorylation of guanosine derivatives to GTP.

The following scheme can therefore be proposed for the cyclic reamination of the adenylic system.

1) Inosinemonophosphate + aspartate + guanosinetriphosphate = adenylosuccinate + guanosinediphosphate + orthophosphate;

2) Adenylosuccinate + H_2O = adenylate + succinate;

3) Guanosinediphosphate + ATP = guanosinetriphosphate + ADP.

The above over-all reaction in muscle and in brain was shown by the present investigation to require PALPO, ATP and an unknown, thermostable, dialyzable factor. The latter factor is believed to be guanosinetriphosphate.

The concept of the reversibility of adenylate deamination gained further support from the recent work of Wajzer et al. [25]. These authors studied the changes in UV absorption maxima of muscle fibers. It was found that during a single contraction the maximum UV absorption fell from a value of 546 to 240 μv and then rose again to the initial value on relaxation. These changes were related to the reversible interconversion of adenylate and inosinate.

SUMMARY

A previous observation on the utilization of dicarboxylic amino acids, and of their deamination products, in the reamination of adenylate in brain and muscle preparations, was studied further, with special reference to the type of interconversion of these amino acids. The experiments were carried out with both brain and muscle homogenates, in media containing potassium and magnesium ions, phosphate, fluoride, adenosinetriphosphate and pyridoxal phosphate.

Incubation of both brain and muscle homogenates, alone, caused an increase in the levels of free amino acids, particularly of the dicarboxylic amino acids. When brain homogenates were incubated in the presence of either glutamate or aspartate, there was an increase in the level of γ-aminobutyrate or β-alanine, respectively. Under similar conditions muscle homogenates dicarboxylated aspartate, but not glutamate. In both brain and muscle homogenates, the addition of ATP stimulated ammonia production from adenylate, and inhibited the hydrolysis of the amides-asparagine and glutamine. A comparison of the rates of glutamine and asparagine hydrolysis with that of adenylate deamination, and also a comparison of the intensity of ammonia formation with the interconversion of the amino acids, suggested that in muscle and brain homogenates, adenylate was reaminated by a cyclic reaction. In this reaction the amino group was donated by glutamate in brain and by aspartate in muscle; the corresponding dicarboxylation products — γ-aminobutyrate and β-alanine respectively — also served as donors.

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EXAMINATION OF THE ELECTROPHORETIC PROPERTIES OF PHOSPHOLIPASE C OF C. PERFRINGENS TOXIN AT DIFFERENT STAGES OF THE DETOXIFICATION PROCESS

M. V. Ispolatovskaya and I. A. Larina

N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences USSR, Moscow

Toxoids prepared by formalin treatment of filtrates of bacterial cultures are used for active immunization against gas gangrene, tetanus or diphtheria. Conversion into toxoid by the action of formalin involves gradual loss of toxicity of the filtrates, with retention of their antigenic properties. In place of their lost toxic properties the protein has now acquired new, protective properties.

The detoxification process is one of extraordinary complexity. Much work has been devoted to the study of this problem, most of it dealing, however, with the elucidation of the mechanism of reaction of formaldehyde with individual amino acid components of the protein molecule [1], but nothing is known about the physical chemistry of the detoxification process.

Uspenskaya and Meduski [2] have shown that preparations of crude toxin can be used for the investigation of the electrophoretic properties of phospholipase C, without the need for preliminary purification, applying the starch-slab electrophoresis procedure. We applied this procedure in our research.

MATERIALS AND METHODS

We conducted our experiments using toxin produced by C. perfringens, strain 28, grown in meat broth culture for 16-17 hr at 37°. The freshly prepared native toxin was concentrated by salting out with ammonium sulfate at 50% saturation, which gave a highly active toxin solution containing 3000-5000 mouse MLD/ml, with a phospholipase activity of 32,000 LV/ml (a LV is a lecithovitellin unit). The phospholipase activity was derived from the lecithovitellin reaction described by Macfarlane [3], and the toxicity, in MLD, from experiments with white mice [4].

Detoxification of concentrated C. perfringens toxin was effected by means of a single addition of formalin to a final concentration of 0.4%. The solutions were then maintained for 60 days at 37°.

Samples of toxin were taken for starch-slab electrophoresis at set intervals of time. The following tests were performed for each sample, and for each of its electrophoretic fractions: protein content, by Lowry's method [5], phospholipase C activity (LV units), and antigenic activity of the toxin and toxoid, as combining units, CU [a combining unit of the least amount of toxin which will combine with 1 AU (antitoxin unit) of a given antiserum].

For measurements of binding of toxin we used standard serum of known titer, prepared by the Tarasevich Institute (TsGNKI). In particular cases we also determined the MLD of the toxin solutions on white mice. These experiments enabled us to observe changes taking place in the electrophoretic mobility of phospholipase C during the process of transformation of toxin into toxoid, and to compare the electrophoretic mobility of phospholipase C with that of toxoid. The location of the toxoid fraction on the electrophoretograms was determined from its antigenic activity. Samples were taken after 1, 2, 3, 4, 7, 10, 15, 28, 35, 45 and 60 days of incubation of the toxin with formalin.

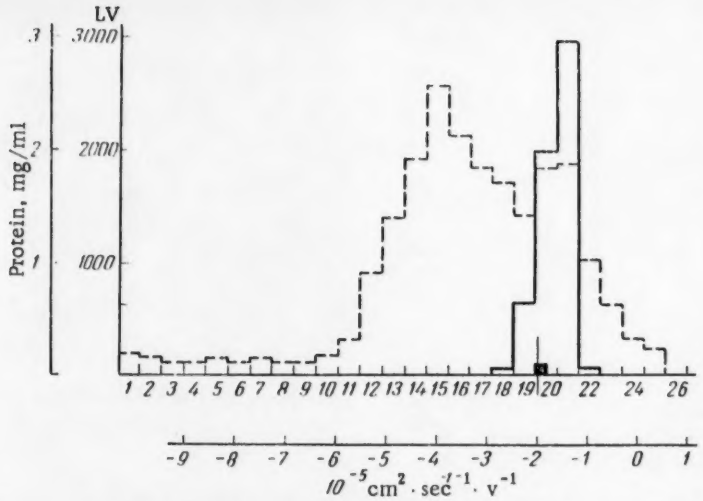

Fig. 1. Electrophoretic fractionation of phospholipase C from C. perfringens toxin. Freshly prepared specimen. Toxicity, 3200 LV/ml; antigenicity, 50 CU/ml. Continuous line, activity in LV units; broken line; protein content in mg. □ – starting point.

Electrophoretic fractionation of the toxins at the various stages of the detoxification process was effected by means of Kunkel and Slater's starch-slab procedure [6]. In the equipment used by us [7], the starch slab was 26 cm long, 13 cm wide, and 1 cm thick. Contact between the slab and the electrode chambers was achieved through paper wicks soaked in buffer solution. Carbon electrodes were used. The electrolyte was borate buffer of pH 8.6, of conductance $\chi = 0.0035$ ohm^{-1} cm^{-1}, with a voltage of 200 v, current strength 45 ma; duration of electrophoresis 19 hr at 4°. At a distance of 7 cm from the cathode, a trench 0.5 cm wide was filled with 6-7 ml of a slurry made up by mixing concentrated toxin solution with washed starch powder. After completion of electrophoresis the slabs were cut up into 25 sections. Each section, representing a separate fraction, was extracted successively with 4 and 3 ml of buffer, collecting the eluate on the filter pump. In some of the experiments the eluate was collected after the starch had settled to the bottom of the tube. Preliminary experiments had shown that two extractions each followed by sedimentation of the starch sufficed for the complete elution of the proteins present in any of the electrophoretic fractions.

We were thus able to evaluate the phospholipase C activity of each fraction, as well as the other forms of activity enumerated above. The summation of all the values so obtained for the individual sections of the starch slab, and comparison of the resulting totals with the values for the initial sample taken for electrophoresis, gave information as to the extent of inactivation of any of the relevant activities during electrophoresis.

The results of electrophoretic fractionation are represented graphically as electrophoretograms. A mobility scale was applied to the graphs. In preliminary experiments we found that, with our technique, there was a linear relationship between the distance traveled by a protein band and the duration of electrophoresis; this was checked by electrophoresis of serum albumin stained with Evans green (T = 1824).

The mobility scale, with its zero point, and the value of each division, was constructed on the basis of data derived from a special calibration experiment, involving starch-slab electrophoresis of a solution containing pure horse serum globulin and human serum albumin, taken in equal proportions. These proteins differ considerably in electrophoretic mobility, and are convenient for the purpose of constructing such scales. The buffer solution, and all the other conditions of electrophoresis applied in this experiment were identical with those for electrophoresis of the toxin solutions.

The electrophoretic mobility of horse γ-globulin in borate buffer of pH 8.6, and of conductance $\chi = 0.0035$ ohm^{-1} cm^{-1}, was determined in a Tiselius apparatus, and was found to be 1.35×10^{-5} cm^2 · sec^{-1} · v^{-1}; the corresponding value for human serum albumin was 5.7×10^{-5} cm^2 · sec^{-1} · v^{-1}.

Yield of Toxin Expressed as Milligrams of Protein and as Lecithinase Units in Fractions Obtained by Starch-Slab Electrophoresis

Introduced		Activity per mg of toxin protein introduced	Isolated in the active fraction		Activity per mg of isolated toxin protein	Yield, as mg protein	Yield, as lecithinase units	Factor by which activity of the preparation has been raised	Inactivation %
protein mg	lecithinase units		protein mg	lecithinase units					
77.0	25 000	324	11.0	8 300	750	79	30	7	70
77.0	17 500	227	8.6	8 260	960	90	50	9	50
66.5	28 000	421	8.5	23 800	2800	100	85	7	15
212	176 000	830	54.6	40 040	733	71	28	4	72

Knowing the electrophoretic mobility of each of these proteins, and their location along the starch slab, it was possible to construct a mobility scale, on which the distance traveled by the protein was expressed in units of electrophoretic mobility.

By applying the scale to the experimental graphs we could readily identify the mobility zone corresponding with that of the protein under examination, viz., phospholipase C from C. perfringens toxin, at different stages of the detoxification process.

EXPERIMENTAL RESULTS AND THEIR DISCUSSION

Fig. 1 shows the results of a typical experiment on the electrophoretic fractionation of phospholipase C from C. perfringens toxin, in a starch slab.

On the abscissa axis we plot centimeters of the starch slab, and applied to this is the scale of electrophoretic mobility in borate buffer of pH 8.6 and $\chi = 0.0035$. The starting point is marked at a distance of 7 cm from the cathode. The noncoincidence of the calculated zero point with that of the application of the protein solution is due to electrosmotic effects, i.e., to movement of the whole layer of liquid through the thickness of the starch slab, due to the electric field. Phospholipase activity, expressed in LV units, is plotted on the ordinate axis.

It is evident from Fig. 1 that most of the phospholipase activity was concentrated in fractions 20 and 21 of the starch slab, counting from the anode. These fractions are distinguished by an electrophoretic mobility of $1.2 - 1.6 \times 10^{-5}$ cm$^2 \cdot$ sec$^{-1} \cdot$ v^{-1}. Considerable amounts of inactive protein were separated during electrophoresis, giving a 7-10-fold purification of the phospholipase.

Considerable inactivation of the enzyme takes place during electrophoresis, the yields of activity amounting to 30-75%.

These findings are illustrated by the numericalvalues of the table..

Figs. 2 and 3 represent the change in electrophoretic mobility of phospholipase C during the course of detoxification of the toxin of C. perfringens by the action of formalin.

Since formalin gradually transforms the toxin into toxoid, the system under investigation could be regarded as a two-component one, containing phospholipase C and a new protein, toxoid, of different physicochemical properties.

Phospholipase activity was evaluated from the lecithovitellin reaction, and from that of binding of antitoxin. The activity of the toxoid was evaluated only on the basis of the reaction of fixation with the specific serum.

Changes in the electrophoretic properties of phospholipase C of C. perfringens toxin were already perceptible after two days of incubation with formalin, and parallel with this there was considerable accumulation of the toxoid product, which we shall term anaphospholipase C. The distributions of the phospholipase and the anaphospholipase activities coincided.

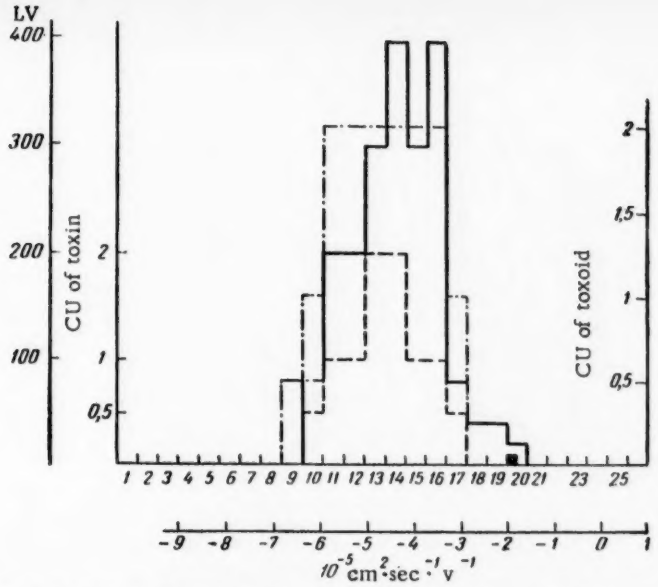

Fig. 2. Electrophoretic fractionation of phospholipase C of C. perfringens toxin four days after addition of formalin. Residual toxicity =3000 LV; antigenicity of the toxoid =80 CU/ml. Continuous line — phospholipase C activity, in LV units; broken line — activity of toxin, in CU; dash-dot line — activity of toxoid in CU; □ — starting point.

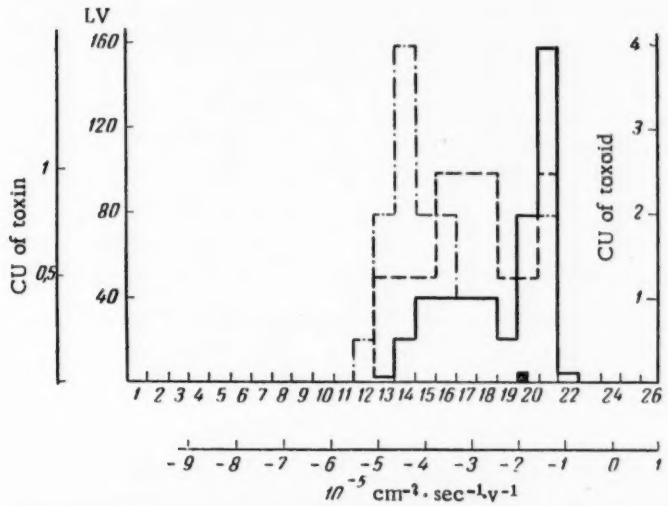

Fig. 3. Electrophoretic fractionation of phospholipase C of C. perfringens toxin ten days after addition of formalin. Residual toxicity 3000 LV; antigenicity of the toxoid = 50 CU/ml. Continuous line — phospholipase C activity, in LV units; broken line — activity of toxin, in CU; dash-dot line — activity of toxoid, in CU; □ — starting point.

The electrochemical properties of the protein changed considerably after four days of incubation; its acidity increased, and the width of the active band rose, covering the range of electrophoretic mobilities from -1.6 to -6.5 x $10^{-5} \cdot cm^2 \cdot sec^{-1} \cdot v^{-1}$ (Fig. 2).

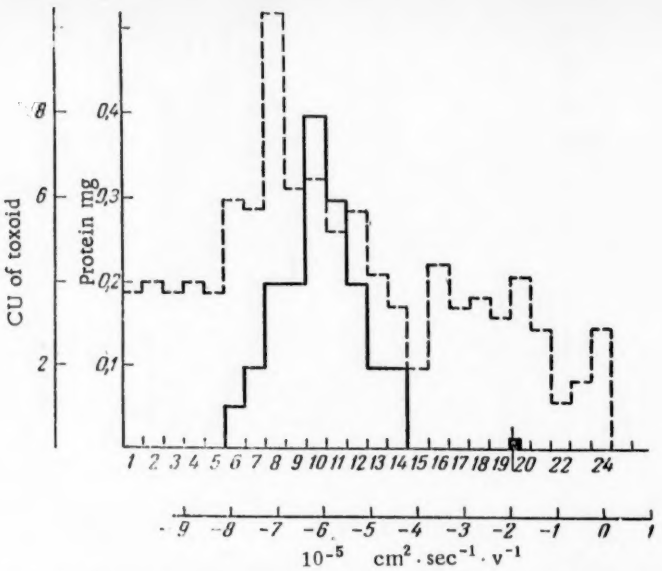

Fig. 4. Electrophoretic fractionation of anaphospholipase C from C. perfringens toxoid 60 days after addition of formalin. Antigenicity of the toxoid = 100 CU; continuous line — activity in CU; broken line — protein content in mg; □ — starting point.

Whereas during the first two days of incubation with formalin, detoxification proceeds very rapidly, only 5000 LV units per ml of toxin solution remaining at the end of this time, as compared with 32,000 units initially, the rate of detoxification falls considerably thereafter. By the end of the fourth day residual toxicity had fallen to 3000 LV units, and by the end of the seventh day, changes in the distribution of the protein along the starch slab were evident, although there was no further change in toxicity.

After ten days of incubation, activity was maximum in fraction 21, and extended as far as to fraction 13 (Fig. 3). The electrophoretic heterogeneity of phospholipase C was also found on the 15th, 28th and 35th days of incubation with formalin. Detoxification was completed on the 60th day.

Fig. 4 represents the distribution of activity along the starch slab, for the final detoxification product. The distribution of protein is not shown in this figure. It is evident, from a comparison of Fig. 4 with Fig. 1, that the electrophoretic properties of phospholipase C differ considerably from those of anaphospholipase C.

Thus our findings show that during the process of detoxification by the action of formalin, the first change is in the electrophoretic properties of phospholipase C, which then undergoes transformation into a qualitatively new product — toxoid (anaphospholipase C). Phospholipase C from C. perfringens toxin is electrophoretically homogeneous, whereas anaphospholipase from C. perfringens toxoid is heterogeneous as regards its protein behavior.

The process of detoxification by the action of formalin appears to involve the gradual modification of phospholipase C protein, which becomes progressively more acidic. During the process, changes occur in the surface density of the charge of the molecules, expressed by a rise in electrophoretic mobility of from -1×10^{-5} to -6×10^{-5} $cm^2 \cdot sec^{-1} \cdot v^{-1}$. Gradual denaturation or blocking of the active centers of the enzyme protein of phospholipase C takes place, not, however, involving that part or structural feature of the molecule responsible for its antigenicity. This process does not proceed simultaneously for all of the protein molecules, but proceeds gradually and selectively, to which may be due the slow rate of detoxification of phospholipase C by formalin.

Uspenskaya and Meduski [2] reported that their specimen of phospholipase C, freshly prepared from C. perfringens toxin in Warsaw, was electrophoretically homogeneous, and had an electrophoretic mobility close to zero.

Quite a different distribution of activity was found when 7-year-old specimens of phospholipase C, pre-

pared by the Central Institute of Experimental Medicine, were subjected to electrophoresis. Instead of the activity being concentrated in a narrow band, it occupied a broad zone, ranging from zero mobility to -9×10^{-5} $cm^2 \cdot sec^{-1} \cdot v^{-1}$.

Our study of the changes taking place in the electrophoretic properties of phospholipase C during the detoxification process suggested that the diffuseness of the active band might be ascribable to spontaneous detoxification, which has often been reported to take place during prolonged storage of C. perfringens toxin. It is evident that the changes in electrophoretic mobility of phospholipase C of C. perfringens toxin occurring during formalin detoxification may to a certain extent be identified with those taking place during spontaneous detoxification and partial conversion into toxoid, associated with aging of the toxin.

Our study of the electrophoretic properties of phospholipase C of C. perfringens toxin revealed a series of intermediate stages of modification of the protein molecule, leading to the gradual loss of one of its basic properties – toxicity – but not of its antigenic and protective qualities. Loss of lecithovitellin activity takes place together with loss of toxicity of the enzyme, while the activity of the toxoid preparation, termed provisionally anaphospholipase C, can only be assessed from the reaction of combination with specific antiserum.

Our experiments showed that a freshly prepared specimen of phospholipase C from an 18-hour C. perfringens culture in meat broth gives a sharp peak of activity. The changes in electrophoretic mobility and in electrophoretic homogeneity of phospholipase C, reported by Uspenskaya and Meduski for specimens obtained from TsIÉM,* appear to be due to their aging since they had been stored for 7 years, and not to be associated with the culture time; no appreciable lysis of bacterial cells is observable at the time at which C. perfringens cultures exhibit the highest toxin production (18 hours).

C. perfringens toxin preparations are always heavily contaminated with pigmented substances whose chemical nature is quite unknown. The active fractions obtained by electrophoretic purification of the enzyme on a starch slab do not contain any of these pigments. The pigmented fractions have a high protein content, but do not exhibit any biological activity. Our experiments showed that the pigments are bound to protein of electrophoretic mobility from -3.7 to -5.2×10^{-5} $cm^2 \cdot sec^{-1} \cdot v^{-1}$. Elimination of pigment is of importance in the preparation of products for active immunization against gas gangrene, and the problem merits further investigation.

SUMMARY

Phospholipase C obtained from a freshly prepared solution of C. perfringens toxin, concentrated by precipitation with ammonium sulfate, is electrophoretically homogeneous, and its electrophoretic mobility is close to -1×10^{-5} $cm^2 \cdot sec^{-1} \cdot v^{-1}$.

Conversion of the toxin into toxoid, by the action of formaldehyde, is associated with change in the electrophoretic properties of phospholipase C; new protein fractions make their appearance, having a range of electrophoretic mobilities of from -1.2 to -6.5×10^{-5} $cm^2 \cdot sec^{-1} \cdot v^{-1}$.

C. perfringens toxoid which is a qualitatively distinct derivative of phospholipase C, differs considerably from it in its electrophoretic mobility, which ranges from -4.5 to -8.5×10^{-5} $cm^2 \cdot sec^{-1} \cdot v^{-1}$.

In conclusion, we wish to express our profound gratitude to V. D. Uspenskaya for the help and advice she gave us during the prosecution of this research.

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THE EFFECT OF RICKETTSIA TOXIN ON OXIDATIVE DEAMINATION OF AMINO ACIDS

L. P. Zubok

The N. F. Gramaleya Institute of Epidemiology and Microbiology, USSR Academy of Medical Sciences, Moscow

The specific intoxication of typhus is one of the chief symptoms of this disease. Suspensions of live rickettsia, obtained from gall bladders of chick embryos, from infected lungs or from infected lice, contain a toxic substance tightly bound to the rickettsial cell and lethal to white mice within 2-6 hours. The toxin may be specifically precipitated by immune sera, it is thermolabile at 60° and can also be destroyed by a variety of chemical agents [1-10]. Rickettsial intoxication is demonstrated in interference with circulation in the capillaries and arterioles of the central nervous system and of some internal organs (hyperemia, paretic vasodilatation, stasis) [11-12]. The pathophysiological mechanisms underlying this intoxication are as yet unknown.

There has been a trend recently in pathology of infections to direct the attention to the problem of bacterial enzymes as the likely pathogenic factors in the development of infective illness. The enzymes of the bacterial cell are capable of catalyzing specific chemical reactions alien to the host organism, and resulting in the formation of compounds towards which the host is far from indifferent, and which may cause severe intoxication of the organism.

The detection and study of such enzymes may help not only in the solution of some of the problems of pathogenesis of infections diseases, but may also help to arrive at a more rational approach to therapy, and lead to the evolement of methods for the cultivation of the various causative organisms, including rickettsia, in artificial media.

Rickettsia harvested from the chick-embryo gall bladder are known to contain the enzymes glutamate oxidase [12-21] and glutamic-aspartic transaminase, but the other transaminases, namely leucine, phenylalanine, valine, arginine and tyrosine transaminase were not detected in this material [19].

Previous reports from this laboratory [21] indicated that Provachek rickettsia grown in the lungs of albino mice could oxidize glutamate, but not glucose, oxalate, lactate or succinate. Calculations of the activation energy for glutamate oxidation revealed the extremely high energy requirement of rickettsia in the enzymatic catalysis of this reaction, indicating the primitive state of rickettsial enzymatic make-up and their dependence on coupling with the enzymatic processes of the host cell. A further insight into the metabolic activity of the intracellular parasite is therefore only possible through a simultaneous study of the interrelations between the microbial and host metabolism. The present work attempts such a study.

METHODS

Albino mice, weighing 10-12 g, were used throughout. Live rickettsia were obtained through multiple differential centrifugation of a suspension of lung tissue from mice previously infected intranasally with Provachek rickettsia [21].

Intoxication was induced in mice by the intravenous injection (into the tail vein) of an absolutely lethal amount of rickettsial toxin contained in 0.5 ml of a suspension of the purified bacteria. Such a dose was lethal in the white mice used within one hour. The toxic effect of the rickettsia did not demonstrate itself before one-

TABLE 1

The Effect of Rickettsial Intoxication on the Oxidative Deamination of Glutamate by Tissue Homogenates of Albino Mice (Incubation time: 1 hour at 37°; Data are in μ g-atoms 300 mg tissue. The values represent means of 4-5 expts.)

Tissue	Oxygen consumption		Ammonia formation	
	without glutamate	with glutamate	without glutamate	with glutamate
Control mice				
Brain	14.1	18.1	1.3	1.9
Liver	26.0	51.6	3.2	4.5
Heart	33.6	117.4	0.95	1.9
Muscle	8.9	13.4	1.1	1.4
Treated mice				
Brain	14.8	23.6	1.8	3.3
Liver	29.3	73.3	3.6	7.0
Heart	34.4	146.6	1.4	3.3
Muscle	11.4	20.7	1.2	2.4

half hour had elapsed from the time of intravenous injection; within one hour the animals became immobilized, developed convulsions and showed terminal respiratory disruption. The animals were killed by decapitation when moribund (5 to 10 mice were used in each experiment). Each of the tissues investigated (brain, liver, heart and skeletal muscle) were shredded in the cold to a pulp, and then ground in a mortar to the consistency of a homogenate.

Tissue homogenates (300 mg) were incubated in Warburg vessels at 37° in 2.5 ml phosphate buffer (pH 7.2-7.3) and in the presence of some amino acid in the final concentration 0.01 M. Incubations were carried out under aerobic conditions. The vessels employed were arranged for the absorption of NH_3, as well as CO_2, so that the intensity of oxidative deamination could be assessed with accuracy. Ammonia was determined according to Conway [22].

Control estimations were carried out with similarly prepared tissue suspensions taken from normal animals which were kept under the same conditions as those infected with the bacteria. In one experimental series, namely, in that designed for the study of the effect of rickettsia toxin on the deamination of glutamate in liver and brain, the control animals used actually received an injection of 0.5 ml of a preparation obtained in the same way as the toxin, but prepared from lungs of normal mice.

All results in the tables are given on the basis of 300 mg tissue.

RESULTS AND DISCUSSION

It was shown experimentally that, in all tissues of treated mice investigated, both O_2 consumption and NH_3 formation rose considerably above that of controls when glutamate was the substrate (Table 1). Thus, in treated animals, as compared with controls, oxygen consumption increased by the following factor: in brain – 2; in liver – 1.75; in heart – 1.3 and in muscle – 2; the corresponding increase in ammonia production was: in brain – 2.3-fold, in liver – 2.8, in heart – 2 and in muscle – 4-fold. Even in the absence of added substrates there was a notable increase above control values in the oxygen consumption and in ammonia production in the tissues of animals poisoned with rickettsial toxin.

Experiments were further carried out (for conditions, see Table 1) to compare the oxidative deamination of glutamate in the tissues of normal white mice and in those of normal mice which have received an intravenous injection of a lung suspension from healthy mice: the results were as follows:

	Normal mice		Mice given 0.5 ml of lung suspension intravenously	
	O_2	NH_3	O_2	NH_3
Brain	4.0	0.67	4.1	0.62
Liver	25.0	1.3	24.1	1.4

Thus, intravenous introduction of a lung suspension from normal mice did not affect the levels of oxidative deamination of glutamate.

In order to determine whether the increase in oxidative deamination tissues of rickettsia-intoxicated mice was specific for glutamate alone, the liver homogenates from treated mice were incubated with each of the following amino acids: L-aspartate, DL-valine, glycine, α-alanine and DL-phenyl-α-alanine (Table 2).

TABLE 2

Oxidative Deamination of Amino Acids by Liver Homogenates From Normal and Rickettsia-Intoxicated Mice (Incubation — 1 hour at 37°. All values in μatoms/300 mg tissue)

	L-aspartate		α-alanine		Glycine		DL-valine		DL-phenyl-α-alanine	
	O_2	NH_3	O_2	NH_3	O_2	NH_3	O_2	NH_3	O_2	NH_3
					Control mice					
	6.4	1,3	5,7	2,7	5,2	0,68	4,0	1,5	4,6	1,5
	10.6	1,4	5,8	2,5	5,2	1,5	3,1	1,4	3,5	1,6
	15,2	1,3	—	—	—	—	—	—	—	—
mean	10,7	1,3	5.8	2,6	5,2	1,1	3,5	1,4	4,0	1,55
					Treated mice					
	5,7	1,3	5,3	2,1	4,5	0,68	3,3	2,0	4,0	1,8
	10,3	1,3	6,0	2,3	4,5	1,4	3,5	1,6	4,4	1,6
	15,3	1,3	—	—	—	—	—	—	—	—
mean	10,4	1,3	5,6	2,2	4,5	1,0	3,4	1,8	4,2	1,7

It follows from the above results that oxidative deamination in liver homogenates in the presence of amino acids other than glutamate was not affected by rickettsial intoxication of the animal.

It was of interest to check whether the deamination product of glutamate — α-ketoglutarate — was also oxidized more intensively in rickettsial intoxication. The oxidation of α-ketoglutarate was studied with muscle homogenates.

It will be seen from Table 3 that, with α-ketoglutarate as a substrate, muscle homogenates from treated animals consumed oxygen at a rate twice that of homogenates from normal mice. The present data thus indicate that rickettsia intoxication in white mice produced a specific activation of glutamate deamination and of the oxidation of α-ketoglutarate.

The activation of the oxidative deamination of glutamate in various tissues of rickettsia-injected mice may be due to a number of reasons:

1) The poisoned animals are in a moribund state when killed. The characteristic property of moribund metabolism is, apparently, the fact that the cells of the animal, particularly those of the brain, derive their energy from glycolysis, rather than from oxidative processes. The intensification of glycolysis brings about a considerable rise in the blood and tissue levels of partially oxidized products [23]. When such tissues are placed into optimal conditions, an increase in oxygen consumption is likely.

2) The effect observed is a sum of activities due to reactions taking place in the rickettsia cells and in the

unchanged host cells.

3) The effect is due to the changes in metabolic pattern of the host tissue induced by the toxic agent of the rickettsia.

A check on the first possible cause showed (Table 4) that in the brainsof moribund animals,oxidative deamination of glutamate proceeded at a rate lower than in those of controls (the animals were killed by air embolism).

Thus, the observed increase in oxygen consumption and ammonia production in rickettsia-intoxicated animals was not due to their moribund state.

TABLE 3

The Effect of Rickettsial Intoxication on the Oxidation of α-Ketoglutarate by Liver Homogenates of White Mice (Incubation – 1 hour at 37°. All values in μ g-atoms/300 mg tissue)

O_2 consumption		O_2 consumption	
homogenate	homogenate + + α-ketoglutarate	homogenate	homogenate + + ketoglutarate
Control mice		Treated mice	
7.4	16.0	11.2	25.0
10.4	21.5	18.2	36.0
7.8	24.6	9.8	31.5
9.1	13.4	17.1	44,9
mean 8.6	18.8	mean 14.0	34.3

In order to check the second assumption, standard rickettsia suspensions (the equivalent of lungs from three mice in 1 ml of Ringer's saline) as well as their 10-, 50- and 100-fold dilutions were incubated in the presence of each glutamate and α-ketoglutarate.

The results of this experiment showed that the rickettsial suspension, already in the 1:10 dilution, failed to use oxygen in the presence of either glutamate or α-ketoglutarate as a substrate.

Calculations showed that the number of rickettsia present in the sample tested in vitro was many times the effective number contained in any of the in vivoincubations [21].

Calculation: 0.5 ml of the basic rickettsia suspension was diluted 30-fold before injecting into a mouse weighing 10-12 g, containing a total of 5 ml of body fluid; the dilution was thus 1:300.

The animal tissues were diluted 5-fold in suspension, and 1.5 ml of the homogenate was diluted to 2.5 ml in the Warburg vessel, the final dilution is thus 300× 5 × 1.5 = 2000.* In the in vitro experiments, 1.5 ml of the basic suspension was used, i.e., three times that used for injection. The final content of rickettsia in the vitro tests was therefore 6,000 × that of the in vivo experiment. It must also be stressed that in the short time interval employed – one hour afterinjection – no significant multiplication of rickettsia could have taken place in the host body.

The results allowed exclusion of the second proposed mechanism for the activation of the oxidative deamination of glutamate in rickettsial toxicosis.

In order to check on the possibility of direct action of the rickettsia on the enzymatic systems of the host, brain homogenates from noninfected mice were incubated in vitro in the presence of the basic rickettsia suspension, and its 1:10 dilution.

The results showed that there was no difference in the degree of oxidative deamination of glutamate between brain homogenates from normal animals incubated alone, and those incubated in the presence of a 1:10 dilution of the rickettsial suspension (Table 5). When the brain homogenate was incubated with the undiluted

*As in original – Publisher's note.

TABLE 4

Oxidative Deamination of Glutamate by Brain Homogenates from Healthy and Moribund Mice (Incubation – 1 hour at 37°. All values in μ g-atoms/300 mg tissue)

	Oxygen consumption		Ammonia formation	
	homogenate	homogenate + glutamate	homogenate	homogenate + glutamate
Control mice				
	18,3	27	3,0	4,1
	20,6	28,6	2,3	3,6
	19,3	27,8	2,5	3,7
mean	19,4	27,8	2,6	3,8
Moribund mice				
	18,2	24,3	2,4	2,8
	18,8	25,0	2,4	2,8
	19,7	25,6	2,1	2,6
mean	18,9	24,9	2,3	2,7

basic rickettsial suspension, and with glutamate as a substrate, there was a slight increase in oxygen consumption although there was no increase in ammonia production. Furthermore, the oxygen consumption under those conditions was less than the sum of the oxygen consumptions of the brain homogenate and of the undiluted rickettsia suspension when incubated separately (Table 5). The latter observation indicates that rickettsia in vitro exert a direct toxic effect opposite to that observed in the whole organism, where the intensification of the oxidative deamination of glutamate was due to a change in the metabolic patterns of the animal tissues, elicited by the action of the toxic agent of rickettsia.

SUMMARY

Rickettsial intoxication was shown to bring about a specific activation of the oxidative deamination of glutamate in various tissues (brain, liver, heart and muscle) of the albino mouse. The changes appeared to be due to the toxin-induced changes of the metabolic patterns of the host tissues.

TABLE 5

The Effect of Rickettsia on the Oxidative Deamination of Glutamate by Mouse-Brain Homogenates (Incubation – 1 hour at 37°. Values in μg-atoms/300 mg tissue)

Oxygen consumption		Ammonia formation		Oxygen consumption		Ammonia formation	
no additions	with glutamate	no additions	with glutamate	no additions	with glutamate	no additions	with glutamate
Basic rickettsia suspension				**Homogenate + basic suspension**			
0	8,7	0,3	1,4	20,9	32,0	2,3	3,0
0	5,7	0,2	0,9	18,7	28,6	2,0	2,7
0	5,1	0,2	0,9	17,3	25,7	2,7	3,7
mean 0	6,5	0,2	1,1	mean 18,9	28,7	2,3	3,1
Homogenate				**Homogenate + suspension 1:10**			
20,6	27,6	2,3	3,0	20,9	27,3	2,3	3,0
20,8	27,9	2,0	2,7	20,8	27,9	2,2	2,9
17,1	23,0	2,7	3,7	18,3	24,9	2,9	3,6
mean 19,6	26,1	2,3	3,1	mean 20,0	26,7	2,4	3,1

Rickettsial intoxication also led to the activation of α-ketoglutarate oxidation. The intensity of oxidative deamination of other amino acids (L-aspartate, α-alanine, glycine, DL-valine, DL-β-phenyl-α-alanine) in the tissues of rickettsia-treated animals was not different from those of normal mice.

In vitro, rickettsia showed an inhibitory effect on the oxidative deamination of glutamate, that is, they showed a direct effect of toxin, opposite to that observed in the whole animal.

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PHOSPHORUS METABOLISM OF THE SUPERIOR CERVICAL SYMPATHETIC GANGLION

M. N. Baranov

Laboratory for the Biochemistry of the Nervous System, I. P. Pavlov Institute of Physiology, Academy of Sciences USSR, Leningrad

The superior cervical sympathetic ganglion of cats affords convenient material for the study of the activities of nerve cells. A large number of physiological researches have been conducted using Bykov and Pavlova's procedure [1] for the perfusion of isolated ganglia. The difficulties encountered in the biochemical analysis of ganglia are due to the need to apply ultramicromethods, in view of the small dimensions of the organ (its wet weight is of the order of 10 mg). In some recent biochemical papers [2-4] inorganic and high-energy phosphate contents were determined in ganglia following prolonged stimulation, blocking of nerve conductivity, hypothermia, etc.

The microanalytical procedures worked out by us for the study of the metabolism of certain phosphorus compounds in the various layers of the cerebral cortex [5,6] were applied in the present research. The object of this research was to determine the content of a number of phosphorus compounds in the ganglion (inorganic phosphorus, ATP, phosphocreatine, phospholipids, phosphoproteins, and others) under different conditions, in order to be able to compare the phosphorus metabolism of this ganglion with that of the brain.

METHODS

The ganglia were frozen in liquid oxygen immediately after their removal from cats, and were weighed on a torsion balance (with a scale weighing up to 10 mg). Lipids were extracted by means of a chloroform – methanol mixture (2:1), according to Folch et al. [7], with minor modifications [8]. We found that this treatment inhibited breakdown of labile phosphorus compounds, as did also fixation in acetone – ethanol mixture, used by us in an earlier research [5]. The acid-solublefraction was separated by treatment of the material with 2.5% trichloroacetic acid. The lipid extract was washed three times on the centrifuge with 0.1 and 0.01 N HCl. The protein residue remaining after five washings with 2.5% trichloroacetic acid and with methanol was extracted with acid chloroform – methanol mixture (0.04 N HCl) for two hours at room temperature, to remove phosphorus-containing components of lipoproteins [9] remaining in the tissue after extraction of lipids by the procedure of Folch et al.

The phosphorus compounds present in the acid-soluble fraction were extracted with isobutyl alcohol after conversion of their phosphoric acid content into phosphomolybdic acid [5]. On the basis of reports in the literature [10] we reduced the sodium molybdate content of the test solutions to a fifth of the previous amount (final concentration 0.25%). With such concentrations, and with the amounts of tissue used by us (5-10 mg of fresh ganglion tissue), full extraction of phosphorus is achieved both in the first stage of analysis (extraction of inorganic phosphorus) and in the second stage (extraction of phosphocreatine phosphorus). In order to assure complete extraction of the phosphorus liberated from hydrolysis of ATP in the third step of the analysis, we added 0.05 ml of 6.2% aqueous sodium molybdate to the hydrolyzates. The reason for this modification to the procedure was to reduce the molybdate content of the solutions to a minimum during hydrolysis, as high concentrations of molybdate yield considerable amounts of blue molybdic acid complexes $MoO_4 \cdot MoO_4$ and others) when boiled and this interferes with the analyses. We were thus enabled to extract ATP phosphorus from N H_2SO_4 solution,

TABLE 1

Content of Phosphorus Compounds in the Superior Cervical Sympathetic Ganglion (In mg-% P. The ganglia were taken for analysis immediately after excision)

Part of the ganglion	Inorganic phosphate	Phosphocreatine	Labile P of ATP	Phospholipids	Phosphoproteins
Ganglia analyzed together with capsule					
Cephalic	—	1,8	8,9	60	46
Caudal	10,6	2,2	6,9	104	43
Cephalic	13,5	1,9	7,5	66	44
Caudal	9,9	3,3	12,0	103	46
Cephalic	12,2	1,2	8,0	91	38
Caudal	8,0	2,3	10,5	74	43
Cephalic	11,3	1,1	7,0	100	43
Caudal	7,7	1,5	9,0	80	47
Ganglia analyzed without capsules					
Cephalic	7,4	3,6	15,0	90	63*
Caudal	8,6	2,9	12,6	79	—
Cephalic	6,5	4,0	15,7	90	62
Caudal	5,5	4,2	16,8	94	63
Cephalic	13,3	1,8	11,2	83	55
Caudal	13,0	0,9	13,2	80	51
Cephalic	9,0	1,5	9,0	85	41**
Caudal	13,4	1,4	12,0	81	59

*Ganglia taken 60 min after excision.

**Capsules not removed.

i.e., immediately after hydrolysis of the samples, without the need for neutralization with ammonia.

The chloroform – methanol extractives (lipids), the acid chloroform – methanol extractives (lipoprotein phosphorus), and the protein residues were digested in the usual way with sulfuric acid – nitric acid mixture. The inorganic phosphate was extracted from each digest with isobutyl alcohol, as phosphomolybdic acid. The usual concentration (1.25%) of molybdate was taken for the test solutions.

Phosphorus was determined by the method of Martin and Doty [11]. The intensities of coloration were measured in a FÉK-M photocolorimeter using a red filter and a cuvette with a 0.3 cm light path. We usually took 1.25 ml of solution for the readings, raising the volume to 2-3 ml for phospholipids, and to 2 ml for proteins. The radioactivity of the solutions was measured after the colorimetric measurements had been completed. Radioactivity was measured with the aid of an end-window counter.

RESULTS

Contents of Phosphorus Compounds in the Ganglia

Applying the above-described analytical procedures, we determined the contents of phosphorus compounds in the brain of rats, frozen in liquid oxygen. The results of these experiments confirmed that these procedures were applicable to the fractional analysis of nervous tissue since the results conformed to those published in the literature. Thus we found that the mean content of inorganic phosphorus in the cerebral hemispheres amounted to 12.5 mg-% (8 experiments), and that of the cortex of the cerebral hemisphere to 8.3 mg-% (mean of 5 experiments); the corresponding values for phosphocreatine phosphorus were 6.1 and 6.8 mg-%, for ATP 15.2 and 14.0 mg-%, for phospholipids 182 and 145 mg-%, and for phosphoproteins (including the lipoprotein fraction) 39 and 39 mg-%.

We found, when analyzing ganglia which had been quickly excised and frozen in liquid oxygen, that the contents of phosphorus compounds of the capsules with their associated nerves differed considerably from those

TABLE 2

Rate of Exchange of Labile Phosphorus of ATP in the Superior Cervical Sympathetic Ganglion. (P content in mg-%; specific activity of P in imp per min/μg)

Duration of perfusion, min	Dose of P^{32} per ganglion μC	Inorganic phosphorus		ATP labile phosphorus		$\frac{b}{a}$ · %
		content	specific activity (a)	content	specific activity (a)	
Experiments without letting of blood						
3,5	23	19,4	1200	12,4	380	32
3,5	30	10,8	1165	14,8	306	37
7	14	11,9	128	14,0	30	24
7	45	16,5	2440	15,2	900	37
7	20	12,3	120	11,6	46	38
7	110	14,2	7480	14,2	1190	15
10	30	13,0	415	12,2	210	50
0	22	14,3	315	9,8	130	41
5	7	15,3	375	11,9	192	51
15	90	16,5	2900	12,6	1370	47
20	14	8,0	80	12,1	33	41
Experiments with letting of blood						
3,5	100	9,5	1220	12,7	230	19
3,5	120	15,8	864	11,8	353	41
7	100	15,7	7900	15,7	7800	99
7	35	18,0	292	8,0	288	98
7	35	19,7	260	9,3	350	74
10	30	16,0	425	11,5	380	89
10	100	17,6	1380	9,0	1100	80
15	20	16,1	193	15,3	172	90
15	20	17,4	694	17,4	540	79
15	7	14,5	19	8,0	17	90
20	7	17,5	20	10,3	20	100

of the ganglia themselves. Thus, for example, the phosphoprotein and ATP contents of the ganglion were about twice as high as in the capsule. The results of parallel analyses of the cephalic and caudal portions of a ganglion were more concordant when the capsule was first stripped from the organ (Table 1). The explanation of this is that the relative proportions of ganglionic to capsular tissue (when this has not been stripped) may vary in different parts of the organ. Our findings show that stripped ganglia have a higher ATP content, and a somewhat more protein than when they are analyzed together with the capsule. In all our subsequent analyses the capsules were first dissected off from the frozen ganglia. Decapsulation was particularly important in experiments involving the use of radioactive phosphorus, since the capsule was always contaminated with considerable amounts of blood of high P^{32} content.

Renewal of Phosphorus Compounds in Ganglia

For experiments on phosphorus metabolism involving the use of P^{32} the circulatory systems of both the cervical ganglia were isolated, by the procedure of Bykov and Pavlova [1]. The blood vessels of the head and neck were ligated and severed, in such a way that the blood from the common carotids was directed only into the ganglia, and then flowed out of the severed occipital or jugular veins. The animals were under urethan anesthesia, which caused a slight lowering of body temperature. The mean rectal temperature was 35°. The ganglia were at the same temperature (as was shown by measuring the temperature of the tissues surrounding the ganglia, by means of a mercury thermometer).

A single injection of radioactive phosphorus (20-100 μC per ganglion), in the form of sodium phosphate in physiological saline (usually 0.1 ml), was made into the common carotid artery. Blood flowing out of the ganglion was collected from the severed vein. In view of the total circulatory isolation of the ganglion, practi-

TABLE 3

Rate of Metabolism of Phosphorus Compounds of the Superior Cervical Sympathetic Ganglion. (P content in mg-%; percentage renewal as percentage of the specific activity of the P of the given compound in relation to that of ATP labile P)

Duration of perfusion, min	Protein residue		Lipoproteins		Phospholipids	
	content	% renewal	content	% renewal	content	% renewal
5	40	0,7	7,5	1,4	65	0,05
5	53	0,6	11,1	1,1	78	—
7	51	0,9	10,2	5,0	111	0,11
7	64	0,8	—	—	91	0,05
7	59	1,0	9,0	2,1	93	0,09
10	53	1,1	12,0	1,9	106	0,11
10	64	0,9	9,7	3,5	96	0,16
15	45	0,8	10,5	2,8	85	0,19
15	60	1,9	11,6	9,0	99	0,46
15	38	1,2	7,5	5,1	94	0015
15	54	1,2	9,0	7,3	92	—
Mean:	53		9,8		92	

cally no radioactive phosphorus entered any of the other organs of the cat. We found that blood samples taken from the iliac vein at the end of the experiment contained very little P^{32}. Both ganglia were used in the experiments.

Although only a single injection of P^{32} was given, we found very little change in the radioactivity of the blood flowing through the ganglia during the experiments. This may be ascribed to the circumstance that only a very small amount of blood flows through the ganglion in unit time. As a result, the P^{32} introduced into the common carotid remains there, since there is only one path available for its outflow — the ganglionic vessels.

In view of the slow rate of penetration of radioactive phosphorus into the ganglion tissue, the radioactivity of the blood remains higher than that of this tissue for a considerable period of time. So as to free the ganglionic tissue of admixture of blood of high P^{32} content, in one of the series of experiments we bled the animals by opening the occipital or internal carotid artery, after perfusion had been completed. Whereas in experiments without blood-letting, the specific activity of ATP amounted after 7 min. to 40-50% of that of the inorganic phosphorus of the ganglion, when the animals were bled this value rose to 80-100% (Table 2). In the former case the admixture of blood of high P^{32} content gave a distorted picture of the true relation between these contents.

Our results show that labile phosphorus of the ATP of the ganglia is completely replaced within 7-10 min. We were thus able to base valid conclusions regarding the rate of renewal of phosphorus fractions on the ratio of the specific activity of the latter to that of labile phosphorus of ganglionic ATP, both for the experiments with blood-letting, and in those without blood-letting. In the experiments not involving blood-letting, the duration of which did not exceed 7-15 min., some admixture of blood ATP to ganglionic ATP, the specific activity of ganglionic ATP could not have been raised sufficiently to have any significant effect on our results.

As is evident from the data of Table 3, the lipoprotein phosphorus content of the ganglia has a mean value of 9.8 mg-%; the corresponding value for phospholipids is 92 mg-%, and of the protein residue (the sum of nucleic acid and phosphoprotein phosphorus) is 53 mg-%. The ratios of the specific activities of lipoprotein, phospholipid, and protein phosphorus to that of labile phosphorus of ATP rises, to a greater or smaller extent, with time. Radioactive phosphorus is incorporated into these fractions at different rates. Of these fractions, the highest metabolic rate is found for lipoproteins, being about ten times greater than for the phosphorus of the protein fraction. It is known that similar results have been obtained for the rates of renewal of these compounds in brain tissue.

DISCUSSION OF RESULTS

Perfusion of the ganglia with the animal's own blood containing radioactive P, as applied in our researches, presents certain advantages over the use of saline solutions. With this procedure, the conditions of supply of nu-

trients and of metabolism of the ganglionic tissues do not differ from the normal ones, and the possibility of causing an edematous state of these tissues is obviated; this could lead to considerable errors in calculating the contents of phosphorus compounds. Our experimental findings show that for the biochemical investigation of ganglia (and, a fortiori, for work involving the use of radioactive phosphorus) consistent results are obtainable only if the ganglia are thoroughly freed of capsular tissues (Table 1).

The contents of inorganic phosphorus and of labile phosphorus of ATP were usually within the range of 12-17 mg-%; these values are about the same as for the brain (Table 2). The phosphocreatine content of ganglia was low. Some of our data suggest that freezing the ganglia in situ, and lengthening the time elapsing between completing the operation and excising the ganglion to 60 min led to higher values being found for the phosphocreatine content (Table 1). It appears that some of the phosphocreatine undergoes decomposition during the operation and subsequent excision of the ganglion.

Certain discrepancies between our results for the contents of inorganic phosphorus, ATP, and phosphocreatine and those of Vysotskaya [3] may in all probability be ascribed to differences in the analytical procedures applied. Thus, this author determined phosphocreatine in the filtrate obtained after precipitation of inorganic phosphate by magnesia mixture, which is known to give incomplete precipitation of inorganic phosphate. The values given in this paper for the phosphocreatine content (6-7 mg-%) should hence be considered as being too high.

The phosphorus contents of the protein (53 mg-%) and lipoprotein fractions (9.8 mg-%) of ganglia are much higher, and of the phospholipid fraction(92 mg-%) lower, than in brain tissue. According to Smirnov and Chirkovskaya [8], the corresponding values for rat brain are 31.3, 4.7, and 222.6 mg-%.

Under our experimental conditions, the admixture of blood to ganglionic tissue amounted to 2.4% on the average. As in our previous paper [5], we based the calculation of this value on the P^{32} contents of the blood and the ganglionic tissues, immediately after injecting radioactive phosphorus into the blood. In view of the slow rate of penetration of phosphorus into nervous tissue, the radioactivity of the ganglion was due chiefly to its blood content.

The rate of incorporation of P^{32} into ganglionic tissue is very low, although higher than for brain tissues [12]. Thus the specific activity of inorganic phosphorus of a ganglion was of the order of about a fiftieth of that of blood 7-15 min after injection of labeled phosphate. The increase in specific activity of ganglionic inorganic phosphorus, due to the presence of blood phosphorus, causes low values of relative specific activity of phosphorus fractions, in particular of ATP, to be obtained (Table 2).

A correction for the blood content of the tissue has therefore to be applied in evaluating data obtained for ATP metabolism in the brain [13, 14]. It was possible, for ganglia, to remove most of the blood of high P^{32} content by bleeding the animal, and to make a direct comparison of the specific activities of inorganic phosphorus and ATP. We found that a time of 7-10 min was sufficient for the equalization of the specific activities of these substances, i.e., for the complete renewal of the labile phosphorus of ATP (Table 2a). We found from special experiments that when blood containing radioactive phosphorus was perfused, subsequent bleeding of the animal for 3.5 min did not lead to any considerable depletion of blood of high P^{32} content from the ganglionic tissues. We therefore consider that the time of 3.5 min found by us for renewal of ATP is too low.

In view of its low content, the results of determinations of specific activity of phosphocreatine are not recorded in this paper, since most of them are not trustworthy.

The time interval elapsing between introduction of radioactive tracer and taking of samples for investigation of the phosphorus metabolism of brain tissues has usually been longer than was practiced in our research. The temperature of the ganglia (35°) was somewhat lower than in studies of brain metabolism. It has been shown by Vladimirov et al. [15] that the rates of renewal of phosphorus compounds of nervous tissue vary considerably, according to the temperature conditions prevailing. These differences render it difficult to compare our data with those cited in the literature for brain tissue. It appears, from the following approximative calculation, that the rate of renewal of phosphorus compounds is of the same order of magnitude in ganglia as it is in brain. It has been shown that 2.1% of the phospholipids of rat brain undergoes renewal in 3 hr [16], and 46.6% of the phosphorus-containing lipoproteins [8]. Hence, for a period of 15 min the corresponding figures would be 0.17 and 3.9%. These figures agree satisfactorily with our corresponding data for ganglia (Table 3).

It appears from our results that the relations between the rates of renewal of phosphorus compounds in ganglia are approximately the same as for brain. Thus, the phosphorus of ganglionic lipoproteins has a high rate of replacement, whereas that of phospholipids is very low; the rate of renewal of the former fraction is between ten and a hundred times greater than for the latter.

SUMMARY

The phosphorus metabolism of ganglionic compounds (inorganic phosphorus, ATP, phospholipids, etc.)has been investigated, using the microanalytical procedures previously developed and applied in this laboratory, for a similar study of the layers of the cerebral cortex. With certain additional modifications, these procedures were found to be applicable to the analysis of the major phosphorus fractions of a sample of 5-10 mg of fresh tissue. Consistent analytical results could be obtained for ganglia if they were first thoroughly freed of their capsule with its adnexa; the phosphorus metabolism of these tissues differs greatly from that of the nervous tissue.

The ganglia were isolated from the general circulation, and were perfused with blood entering from the common carotid, so that their conditions of blood supply and metabolism closely approached the normal ones. Introduction of radioactive phosphorus into the carotid blood made possible the determination of the contents of the various phosphorus compounds of the ganglia, as well as the rate of their renewal.

It was found that ganglia contain about as much inorganic phosphorus and ATP as does the brain; its protein and lipoprotein phosphorus contents are higher, and its phospholipid phosphorus content lower, than in the brain. The rate of renewal of phosphorus compounds in ganglia is of the same order of magnitude as in the brain. All of the labile phosphorus of ATP is replaced in ganglia within 7-10 minutes.

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AMPEROMETRIC TITRATION OF SH-GROUPS OF GLYCININ

T. I. Smirnova, B. F. Poglazov and V. L. Kretovich

A. N. Bakh Institute of Biochemistry, Academy of Sciences USSR, Moscow

In our earlier papers [1, 2, 3] we showed, on the basis of ultracentrifugation studies, that soy-bean glycinin is capable of reversible association and dissociation, and that is consists of identical subunits (mol. wt. 20,000), which make up the monomer (S_8 component, mol. wt. 126,000) which associates further, to yield the macromolecules (oligomers) S_{11} component (mol. wt. 240,000) and S_{14} component (mol. wt. 380,000).

The sedimentation diagram of solutions of glycinin in neutral 10% NaCl show two separate components, with S values of 14.0 and 8 Svedberg units. Reversible dissociation to submolecules takes place at pH values lower than the isoelectric point, above which association takes place, giving the S_{14} component. Similar results were found using a Tiselius electrophoresis apparatus. The state of association of glycinin also depends on the ionic strength of the solutions. The readiness with which reversible association takes place is evidence of the existence of intramolecular forces, other than covalent ones, which are responsible for the structure of the glycinin macromolecule.

Our observations are readily explicable on the basis of our proposed mechanism of reversible association of glycinin [3]:

1) At pH values below the isoelectric point there is a reversible equilibrium between the subunits and the monomer, due to the antagonistic actions of Van der Waals forces and electrostatic forces, and being also dependent of steric factors;

2) At pH values above the isoelectric point the Van der Waals forces and the electrostatic forces act in the same direction, thus favoring formation of oligomers;

3) Increase in the ionic strength of the medium abolishes the major part of the electrostatic effects, as a result of screening of the charges of ions of opposite signs.

Briggs and Wolf [4] have also arrived at the conclusion that the configuration changes taking place when soybean globulin is precipitated in the cold are ascribable to electrostatic forces.

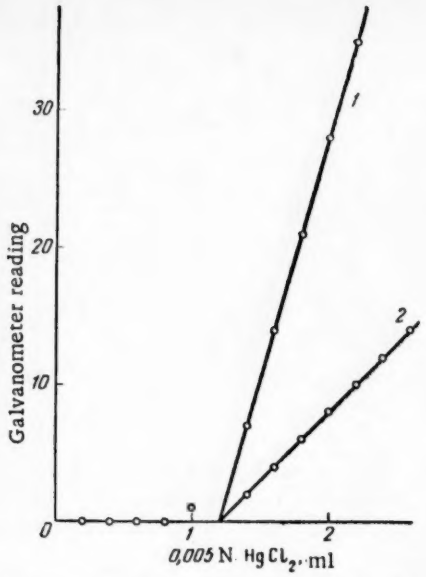
The mechanism of the dissolving action of cysteine on the protein has not yet been elucidated; the S_{14} components undergoes dissociation to the S_{11} component (mol. wt. 245,000), which reverts to the S_{14} component when the solution is dialyzed, or when NaCl is added to it. The ease with which the effect of cysteine on the protein can be reversed is evidence of the high lability of the cysteine – protein complex, and suggests that the effect is also ascribable to modification of electrostatic forces. The action of cysteine is, however, due to its SH-group, since other amino acids have an incomparably smaller peptizing effect on glycinin.

The action of cysteine may be on disulfide bonds. We hence thought it necessary to examine the behavior of the SH-groups of glycinin in the equilibria:

$$S_3 \rightleftharpoons S_8 \rightleftharpoons S_{14}$$

EXPERIMENTAL

Glycinin was examined in alkaline solution (borate buffer of pH 9) and in acid solution (citrate – phosphate



Amperometric titration of SH-groups of glycinin. 1) 500 mg of glycinin, 0.5 M KCl, citrate – phosphate buffer of pH 3.0; 2) 500 mg of glycinin, 0.05 M NH_4NO_3, 0.5 M KCl, borate buffer of pH 9.0.

buffer of pH 3).

Sulfhydryl groups were determined by amperometric titration with mercuric chloride, and in some cases with silver nitrate using the equipment described in an earlier paper [5]. A saturated barium electrode was taken as the reference electrode. For the determination of the number of SH-groups in associated and dissociated glycinin we performed the amperometric titrations at pH 9.0 and 3.0, respectively (see Figure).

As is evident from the figure,, the results of the titrations were the same in both cases. The same results were obtained for solutions of glycinin in 48% urea or in 18% guanidine hydrochloride, in which glycinin is in the dissociated state [4]. Our results are evidence that, even if SH-groupstake part in the polymerization of glycinin, formation of disulfide bridges does not take place, since this would have involved a fall in the number of free SH-groups. Titration of a number of specimens of glycinin with mercuric chloride gave a value of ~ 0.011 μ moles (ranging from 0.010 to 0.012 μ moles) of SH-groups per mg of protein. The same result was given by titration with silver nitrate.

SUMMARY

It can be concluded from our experiments that reversible association of glycinin proceeds without formation of disulfide bridges. This is evidence of the correctness of the mechanism previously advanced by us for the reversible association of glycinin [3]. The action of cysteine on the protein appears to be due to modification of electrostatic forces leading to changes in the relations between these and Van der Waals forces.

We may here postulate some form of specific bonding of the cysteine with the protein, since it is dependent on the presence of the SH-group of cysteine, and the ionization of additional carboxyl groups may lead to coulombic repulsive forces entering into play, preventing addition to the dimer (S_{11} component) of a third monomer molecule (S_8 component), necessary for formation of the trimer (S_{14} component).

Reversible association of reserve proteins of a number of plants has been reported [6-15], and it appears to be an important property of such reserve proteins, in connection with the ripening and germination of seeds.

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NEWS OF FOREIGN SCIENCE

DEVELOPMENT OF BIOCHEMICAL RESEARCH IN THE CHINESE PEOPLE'S REPUBLIC

Wang Ying-Lai

Director of the Institute of Biochemistry of the Chinese Academy of Sciences, Shanghai

Biochemical research began to be prosecuted in China over 30 years ago, and its expansion was greatly stimulated by the publication of the Chinese Journal of Physiology (the first issue of which appeared in 1927). However, under the reactionary Kuomintang regime, biochemistry, in common with other branches of science, developed only very slowly.

Systematic work was conducted only in Wu Hsien's laboratory in Peiping, on a number of subjects, including clinical biochemistry, denaturation of proteins, immunochemistry, and nutrition. Biochemical research was also conducted sporadically in other centers, but these reseraches were few in number, and were of an episodic nature. In general, they dealt only with nutritional problems, which led to the one-sided development of biochemistry in this direction.

During the period of Japanese occupation, from 1937 on, research work practically ceased to be conducted in China. This state of affairs persisted until the liberation of China from the Kuomintang regime.

After the liberation, and with the full support of the Chinese Communist Party, biochemistry, together with other domains of science, entered on a period of intensive growth.

Biochemical reserach is now being conducted on a wide front throughout the country, and many of the current problems of modern biochemistry are studied. Both scientific research institutes and educational establishments are taking part in this work.

There are a number of establishments of the Chinese Academy of Sciences alone, which are taking an active part in the attack on problems of biochemistry. One of the first establishments in which biochemical reseatch began to be conducted is the Institute of Biochemistry (formerly part of the Institute of Physiology and Biochemistry). This Institute is now the leading scientific center for biochemistry; the main subjects of study are enzymes, proteins, nucleic acids and vitamins. One of the more important items of research in the Institute of Physiology is the study of brain metabolism. Researches are being conducted in the Institute of Plant Physiology on photosynthesis, the mechanism of starch synthesis, the physiology of storage of agricultural products, and the biochemistry of microorganisms, in particular with relation to antibiotics. The independent Laboratory of Plant Physiology in Peiping is conducting studies on plant respiration, with special reference to rice. The basic problem studied by the biologists and biochemists of the Shanghai Institute of Experimental Biology is cancer. The research work of the Peiping Institute of Experimental Biology is centered mainly on the problems of the biological functions of nucleic acids. The biochemistry of microorganisms is being studied at the Institute of Organic Chemistry. The staffs of the chairs of biochemistry of the universities and medical schools are engaged in the investigation of a wide range of biochemical problems. Research into problems of medical biochemistry is being prosecuted in the establishments of the Academy of Medical Sciences. We shall here briefly review the work being done by Chinese scientists on the more important problems of modern biochemistry, such as metabolism (including the metabolism of amino acids, proteins, nucleic acids, and vitamins), enzymology, and protein chemistry and biochemistry.

Metabolism. Work on amino acid metabolism has dealt chiefly with that of tryptophan, in particular with its transformation into nicotinic acid (NA). After Henderson had shown that 3-hydroxyanthranilic acid undergoes conversion in rat liver into quinolinic acid [1, 2] it was first established by Chinese scientists that tryptophan is converted by rat liver slices into NA-derivatives, chiefly into quinolinic acid.

It was found that converson of tryptophan into NA is lowered in vitamin B_6 deficiency, but that quinolinic acid production rises. This showed that quinolinic acid is not a direct precursor of nicotinic acid. Addition of pyridoxal phosphate lowered the quinolinic acid content to the normal levels. Vitamin B_6 is inactive in this respect [3].

Vitamin B_6 deficiency also depresses adaptive formation of tryptophan peroxidase in rat liver, induced by administration of excessive amounts of tryptophan to the organism. Tryptophan peroxidase is powerfully inhibited by certain amino acids, viz., phenylalanine, tryosine, cystine, and cysteine. The inhibition is of the competitive type for the first two amino acids. The activity of this enzyme appears to depend on the presence in it of free SH-groups [4]. Contrary to Dalgliesh's findings, it has been shown that vitamin deficiency does not affect transformation of tryptophan into kynurenine [5].

One of the stages in the conversion of tryptophan into nicotinic acid involves the hydroxylation of kynurenine, to yield 3-hydroxykynurenine. It is very difficult to provide direct evidence of the presence of 3-hydroxykynurenine in rat liver, probably owing to the presence of active kynureninase and kynurenine aminopherase. By inhibiting the activity of these enzymes, in vitamin B_6 deficiency, formation of 3-hydroxykynurenine from kynurenine could readily be demonstrated in rat liver slices. Partial purification, with tenfold concentration, of the enzyme present in rat-liver mitochondria is responsible for the introduction of the OH-group into kynurenine. This enzyme is inhibited by versene, but not by α,α'-dipyridyl, o-phenanthroline, or Na-diethyldithocarbamate. Removal of the versene by dialysis reactivates the enzyme, and addition of Mn^{++} or Mg^{++} still further activates the enzyme, as compared with its activity before dialysis.

It has been shown that the activity of this enzyme in mitochondrial preparations from the liver of riboflavin-deficient rats is less than for control preparations, suggesting that riboflavin may be a component of this enzymatic system.

A rapid spectrophotometric method for the determination of 3-hydroxykynurenine in the presence of kynurenine was developed in conjunction with these researches. This method depends on the displacement of the absorption maximum of 3-hydroxykynurenine from 305 to 315 $m\mu$ when the pH of the solution is adjusted to 6.

A study of the dynamics of the enzymatic activity of the liver of rats on a protein-free diet, followed by normal feeding, showed [7, 8] that the leucine aminopeptidase, glutamic dehydrogenase, and glutamic – aspartic acid transaminase activities are lowered during protein deprivation to about the same extent as is the protein content of the liver, and that these activities rise together with the rise in protein content of the liver during subsequent protein feeding. The activities of alanine – glutamic acid transaminase, xanthine oxidase, and uricase fell more rapidly than did the protein content of the liver, and their recovery lagged behind restitution of the protein content. Cathepsin activity was variable, both during protein deprivation and during normal feeding. Addition to the protein-containing diet of 4% of methionine accelerated the process of recovery of enzymatic activity, except for leucine aminopeptidase and cathepsin; the enzymatic activities began to rise within two days of renewal of protein feeding, although the animals were still losing weight. It was shown that the effect of methionine was not due to its sulfhydryl group, or to its methyl group, but rather to the molecule as a whole.

Attempts were made to ascertain whether pathways of amino acid catabolism, other than that established by Braunshtein, exist in the animal organism [9]. It was found that in vitamin B_6 deficiency the urea output of rats was not lower than that of the control animals. It follows from this finding either that rats possess pathways for the production of ammonia from amino acids, other than by transamination, with subsequent deamination by the action of glutamic acid dehydrogenase, or that the residual transaminase activity suffices for metabolizing amino acids entering from the alimentary tract. No differences could be perceived in the urea output of avitaminotic and normal rats when the protein intake or amino acid loading of the animals were raised. Another interesting fact was observed: when food protein of high biological value (ovalbumin) was fed, vitamin B_6 could be eliminated from the diet without causing any appreciable effect on the body weight of the animals, and the appearance of symptoms of vitamin B_6 deficiency was greatly delayed [10].

In connection with the study of the metabolism of nucleic acids and their components, a comparison of the desoxyribose phosphate aldolase activity of the viscera of eight species of mammals and of two species of birds showed that the highest activities were in the spleen of mice, rats, and chicks. A more detailed study of this enzyme, prepared from rat spleen, showed that it resembled the analogous enzyme from E. coli in certain respects, and that it catalyzes the reversible reaction of synthesis and hydrolysis of desoxyribose phosphate [11].

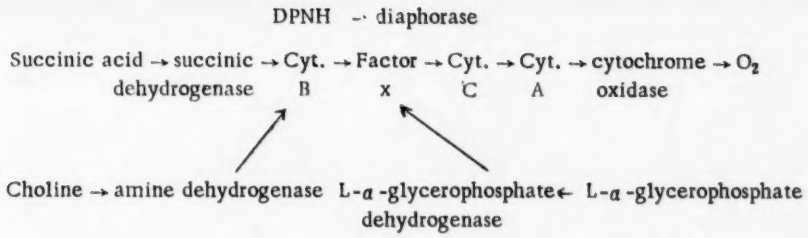
A nucleosidase isolated from a species of Pseudomonas was found to act only on the pyrimidine nucleotides cytidine and uridine [12]. A study was made of rat-liver apurinicase [13] and of swine pancreas ribonuclease [14]. The purity of the former enzyme was raised 15-fold. It is active only in the presence of ions of metals, and has no action on RNA and DNA. Except for certain of its physical properties, the ribonuclease of swine pancreas resembles that of cattle.

New data have been obtained relating to the biosynthesis of vitamin C in the animal organism [15, 16]. It was found, in particular, that the enzyme system involved in the conversion of L-gulono-or L-galactono-γ-lactone into ascorbic acid is located chiefly in the microsomes. A method was developed for the purification of this system, giving a 5-6-fold increase in its activity. A detailed study of this enzyme system showed that its activity was considerably depressed under conditions of riboflavin deficiency. This is evidence of the effect of riboflavin on the activity of the system in question.

Enzymes. Work in enzymology has been directed chiefly to the study of enzymes catalyzing oxidation-reduction reactions, although a number of researches have also been devoted to the study of hydrolases.

The purification of succinic dehydrogenase, and the identification of its prosthetic group, were achieved by Chinese workers [17-19], at the same time that this was done in America by Singer (1954-1955). Their purification procedure differed significantly from that of Singer; it involved dissolution in n-butanol, followed by adsorption on calcium phosphate gel, and fractional precipitation with ammonium sulfate. The product was electrophoretically practically homogeneous. It had an activity of Q_{O2} = 31,000 (determined with phenazine methosulfate as substrate, 1 ml/hr per mg of protein), this activity being nearly twice as great as that of Singer's most active preparations. The purified enzyme contained riboflavin and inorganic iron. Its molecular weight (on the basis of one molecule of riboflavin per molecule of enzyme) was about 140,000. The enzyme does not give any fluorescence when exposed to ultraviolet light, but after digestion with trypsin and chymotrypsin the product gives a strong fluorescence, which is maximum at pH 3. In distinction from other known flavoprotein enzymes, its prosthetic group cannot be detached by any of the ordinary procedures. The enzyme is resistant to boiling in dilute acid solution, which is evidence of the difference between the nature of the bond joining its prosthetic group to the apoenzyme and that present in the other known flavoproteins. After detachment from the apoenzyme the prosthetic group still remains attached to a peptide chain. Analysis of the detached portion showed the presence of flavinadenine dinucleotide (FAD), in addition to peptides. Some idea of the nature of the bond joining the prosthetic group to the apoenzyme was given by the following observations: 1) The absorption maximum of flavin mononucleotide (FMN) and of FAD is at 375 mμ, and is shifted to 355 mμ at pH 12, whereas the flavin peptide from succinic dehydrogenase has an absorption maximum at 350 mμ, which is only slightly displaced when the pH is adjusted to 12. The shift in the absorption maximum of FMN and FAD at pH 12 is probably due to enolization of the keto group at positions 2 or 4, leading to a rearrangement of the double-bond system of the isoalloxazine ring. The absence of such a shift in the case of the flavin peptide at pH 12 suggests that combination with peptide stabilizes the enol structure of the compound; 2) irradiation of the flavin peptide, at any pH, yields a product which is not extractable by chloroform from acid solutions. These findings suggest that the peptide residue is attached to the isoalloxazine ring, although the precise position of its attachment, or the nature of the bond, remain to be elucidated.

Work has been done on the relations existing between cytochrome C and certain dehydrogenases, including succinic dehydrogenase, choline dehydrogenase, reduced coenzyme I dehydrogenase, and α-glycerophosphate dehydrogenase [20-22]. The method used in these researches was based on the competition between two or more of these enzymes for some common, rate-limiting factor. The results obtained are illustrated by the following scheme:


DPNH → diaphorase
Succinic acid → succinic dehydrogenase → Cyt. B → Factor x → Cyt. C → Cyt. A → cytochrome oxidase → O_2
Choline → amine dehydrogenase
L-α-glycerophosphate dehydrogenase
L-α-glycerophosphate

Work on other enzymes has also been done. A new pyridine nucleotide transhydrogenase has been isolated from beef liver, catalyzing the reaction

$$TPNH + DPN^{+} \rightleftharpoons TPN^{+} + DPNH$$

This enzyme has a pH optimum at 8.2, and it differs from the transhydrogenase of Kaplan et al. [23], the pH optimum of which is at 6.2.

The activating effect of certain organic solvents, in particular of acetone and the lower monohydric alcohols, on the oxidation of catechol or pyrogallol has been shown; this effect is greatest for 50% acetone or 50% ethanol. Acetone gives an activation of nearly 300%.

Comparative studies of xanthine oxidase preparations from different animal species have been conducted, in relation to their capability of reacting with various hydrogen acceptors. It was found that the xanthine oxidase of uricotelic animals, such as birds, snakes, and lizards is able to react with DPN, whereas that of nonuricotelic animals, including some reptiles, amphibiae, and fishes, does not react with DPN. In general, the xanthine oxidase activity of uricotelic animals is greater than is that of mammals, which is evidence of the importance of this enzyme to uricotelic animals [24].

Work has also been done on the kinetics of the action of xanthine oxidase. It was found that part of the enzyme – xanthine complex formed from the enzyme – hypoxanthine complex is further oxidized to uric acid, without intermediate dissociation of the complex to free enzyme and xanthine [25].

A procedure for the rapid preparation of cytochrome C of high iron content (0.43%) has been developed. It was found the cytochrome C prepared by this method from yeast consists of two components [26, 27]. An examination of the effect of different bile acids on the succinic oxidase system showed that mono- and di-hydroxycholic acids exert a very powerful inhibitory action. The blocking effect of the inhibitors is obviously exerted between cytochrome C and cytochrome B [28].

Researches on hydrolases included studies of the acid phosphatases of yeast and beans [29-32], and also of soy-bean phytase. An electrophoretically homogeneous preparation of this enzyme was prepared; a study of its reaction kinetics, and of other of its properties showed that it is a single substance, able to act on phytic acid as well as on other acid phosphatase substrates [33].

Proteins. Chinese biochemists have for a number of years been engaged in an intensive study of tropomyosin isolated from different types of muscles of animals of different levels of phylogenetic development. Tropomyosin was prepared in a crystalline state, and electrophoretically homogeneous preparations were examined [34, 35]. Some specimens of tropomyosin contained 16-17% of RNA. Since, however, the protein and the RNA migrated independently of each other in the electric field, it was concluded that a specific nucleotropomyosin complex is not formed [36]. It was shown that the molecular weight of tropomyosin, its solubility, capability of polymerization, crystalline form, and content of loosely bound ribonucleic acid vary to greater or lesser extent according to the source of the specimen.

The molecular weight differences are of special interest, since it is known that proteins of the same kind, derived from different sources, have the same molecular weight, or, at least, belong to one and the same system. This is not the case for tropomyosin. The molecular weights of the monomeric forms of tropomyosin derived from the crop of ducks, pig's heart, shrimp muscle, cephalopod muscle, and rabbit muscle, are, respectively, 150,000, 89,000, 77,000, 68,000, and 53,000. It is possible that the different tropomyosins are structurally very similar, but that they differ in chain length.

Tropomyosin does not possess N-terminal amino groups [37], and it has only one C-terminal amino acid, which is either leucine or isoleucine. This is preceded either by the pair leucine – isoleucine, or by serine – threonine.

Isolation of the corresponding dipeptides, by means of partial hydrolysis, showed that the tropomyosin molecule consists of a single polypeptide chain [38]. The tropomyosin structure recently advanced by Laki and Komintz (according to which the tropomyosin molecule consists of two chains) must therefore be rejected. The absence of proline and tryptophan, the unusually high proportion of polar side groups (61-63%), the tendency towards polymerization and crystallization, its high axial asymmetry (> 20), and its stability necessitate the inclusion of tropomyosin into a special class of proteins. Modification of the side-groups of tropomyosin by means of specific reagents or enzymes, such as arginase, showed that the linear polymerization of tropomyosin involves the amidine, but not the ϵ-amino groups of the chain [40]. It is possible that the polymerization process is due not only to electrostatic forces, with the participation of arginine residues, but also to SH-groups. Rabbit tropomyosin in which the SH-groups were protected at all stages of the purification process showed a considerably higher polymerization capacity.

Considerable work has been done [41, 42] on the isolation and examination of structural myofibrillar proteins, other than actin, myosin, and tropomyosin. At least three other components were found in rabbit skeletal muscle. One of these was a globular protein [43] of isoelectric point pH 4, and with an amino acid composition resembling that of actin. Similarly to actin, this protein did not contain N-terminal amino groups. However, the sedimentation constant was 6.3, in distinction from the value of about 3 for actin. The C-terminal sequence, elucidated by the use of carboxypeptidase, was alanine – tyrosine – leucine, whereas, that of actin is histidine – leucine – phenylalanine. The molecular weight, based on sedimentation and diffusion data [44], was found to be 140,000. The other two components, which also have a lower rate of migration in a Tiselius apparatus than does tropomyosin, at pH values above the isoelectric point, have a sedimentation constant of about 2.3, and do not exhibit any aldolase, creatine phosphokinase, lactic dehydrogenase, or α-phosphoglyceraldehyde dehydrogenase activity. One of them crystallizes in the form of needles, and the other gives spindle-shaped crystals. The latter undergoes reversible polymerization and depolymerization according to the ionic strength of the solutions, and is a complex of thermostable tropomyosin with a second thermolabile protein with a pyridine nucleotide prosthetic group. The molecular weight of the complex, derived from osmotic pressure measurements, is of the order of 70,000. Since these protein components were found in skeletal, cardiac, and smooth muscles, it seems that they are not specific to striated muscle tissue.

Other protein systems have also been investigated. Thus, the molecular weight of procollagen has been determined [45], albumin has been isolated from skin [46] and from the sciatic nerve [47], and it has been shown, by the use of electrophoretic and immunological methods, as well as by comparison of the amino acid compositions and the N-terminal amino acids, that these albumins are identical with serum albumin. These researches provided confirmation that skin albumin is able to diffuse freely through the capillary walls, in the form of an ultrafiltrate. Two lipoproteins have been isolated from brain tissue, and obtained in an electrophoretically pure state. Their chemical and physicochemical properties are now being actively investigated. The review of some of the achievements of Chinese biochemists presented above is evidence of the rapid development of biochemistry in China, and of her growing importance in world science.

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A CENTENARY DATE

V. I. PALLADIN AND THE PRESENT-DAY THEORY OF BIOLOGICAL OXIDATIONS

(On the 100th Anniversary of his Birth)

It is now over half a century since the publication of V.I. Palladin's first papers on the biochemistry of the respiratory process (1908-1909). These papers were published at a time when biochemistry was only beginning to emerge as an independent science, and some time after Bakh had put forward his peroxide theory of oxidation.

An embittered polemic was at that time in progress, regarding the basic pathways of oxidation of carbohydrates, fats, and proteins in living organisms. The protagonists in this conflict of views often displayed uncritical enthusiam for their own theories and hypotheses, while frequently ignoring the indisputably established findings of their adversaries. From these different approaches to the problem emerged the presently accepted theory of biological oxidations, which, although not fully elucidating all the aspects of the problem, does at least provide a finite over-all picture of the complex phenomenon of respiration. It is only fair to point out that the basic principles of this theory had, in their general form, been formulated by V. I. Palladin at the beginning of this century, and were based on the classical researches of A. N. Bakh on the oxidative enzymes of plants. It was not until the appearance of V. I. Palladin's papers, which were based on the results of simple, well-planned experiments, and on a profound theoretical analysis of the problem, that the concept of oxidation of carbon by molecular oxygen was definitively disproven, and that "the hydrogen era of the study of the biochemistry of respiration," to quote V. I. Palladin's expression, was inaugurated.

Further developing the view that hydrogen is the sole oxidizable constituent of organic substrates in the respiratory process, and six months before the publication of Wieland's carefully worked-out theory (1912) of activation of hydrogen and of dehydrogenation – hydrogenation reactions, V. I. Palladin enunciated the basic principles of this theory. He termed the enzymes responsible for activation of the hydrogen of oxidizable substrates "reductases," whereas Wieland called them dehydrogenases. Unfortunately, certain fundamental errors were introduced in the course of the general development of Wieland's theory; V. I. Palladin was able to avoid these errors, thanks to his scientific perspicacity and to the importance which he attached to the biological aspects of the problem, as well as to its chemical ones. Wieland continued for a long time to uphold the view that the only enzyme concerned in the complex process of biological oxidation was dehydrogenase. All that was needed was for this enzyme to activate ("loosen") the hydrogen of a substrate, when it would break loose from the substrate, and would react directly with any acceptor, including molecular oxygen. Thus Wieland's theory, although it represented a major advance in the history of modern biochemistry, did not sufficiently take into account the activation of oxygen, which had already been established by the work of A. N. Bakh. V. I. Palladin succeeded in overcoming this difficulty. In his brilliantly worked-out theory of respiratory "chromogens" V. I. Palladin showed that the process of splitting off of hydrogen from an oxidizable substrate and of its transfer to activated molecular or peroxidic oxygen in living organisms involved the participation of a series of intermediate hydrogen (or electron) carriers, which he termed "intermediaries."

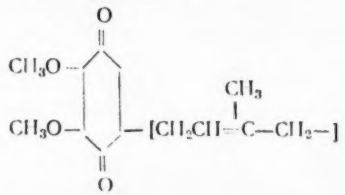
There is full justification for the statement that these papers of V. I. Palladin, and the conclusions therefrom which he advanced with his characteristic force, have served as a basis for the presently held views on the chemical nature and the biological role of intermediary catalysts in processes of respiration. At the level of development of chemistry, and particularly of biology, prevailing at that time, it was natural that V. I. Palladin's attention was directed towards those intermediary catalysts which were the most readily available. These were

the polyphenols, which are very widely distributed in the plant world, mostly in the form of glycosides, but less frequently in the free form. V. I. Palladin called these combined polyphenols "prochromogens," from which, by the action of glycosidases, which to some extent fulfil a regulatory role in the respiratory process, chromogens, i.e., free polyphenols, are formed.

The same respiratory pigments (quinones) are produced by oxidation due to polyphenoloxidase or to peroxidase, and they are able reversibly to accept hydrogen from respiratory substrates, and then to transfer it to oxygen. Thus, V. I. Palladin's theory, based on the principle of dehydrogenation of the substrate with subsequent transfer through intermediaries to oxygen, this process being activated by an enzyme, contains all the basic elements of the present-day theory of biological oxidations. Although V. I. Palladin believed that the chief carriers of substrate hydrogen in plants were the lower phenols, he nevertheless envisaged the existence of other carriers. Thus, he pointed out the importance of aromatic compounds in the respiratory process, and he foresaw the role of phosphorus compounds (1912). It was not until over 20 years later that the role of phosphopyridine nucleotides as hydrogen carriers was discovered. Soon after this it was found that flavin compounds acted as mediators.

It would have been practically impossible to identify such compounds with the aid of the chemical and physical means available in the first decades of the 20th century. The mediatory role of the lower polyphenols, or, more precisely, of their corresponding quinones, could be demonstrated by the aid of simple methods. In connection with A. N. Bakh's well-known papers on the chemical nature and the biological role of polyphenoloxidase, , V. I. Palladin developed a simple and elegant theory of biological oxidation, according to which polyphenoloxidase fulfilled the role of an enzyme completing the oxidation process (a terminal oxidase). With the further development of biochemistry, however, and in particular after the discovery of the cytochrome system, present in all aerobic cells, the role of polyphenoloxidase as a terminal oxidase, not only of animals, but also of plants, began to be doubtful. Initially, these reservations were aroused only by the findings that all plant cells contained cytochromes, but with time other objections arose. The mere fact that cytochromes are universal components of plant cells would not in itself afford decisive evidence of their exclusive, or even major, role in plant respiration. Palladin's "chromogens" are equally widely distributed, as are also Bakh's polyphenoloxidases. However, with increased insight into the mechanism of the respiratory process these objections became more founded. In this connection, an important finding was the absence of polyphenoloxidase from mitochondria, in which the basic respiratory function of cells is known to be located. Moreover, the relation of the polyphenoloxidase system to oxidative phosphorylation is not clear. However, notwithstanding the increasing number of objections to this theory, it might perhaps be premature to delete this powerful enzyme system of plants from the list of terminal oxidases. Even should this sytem not be a terminal one, the biological role of V. I. Palladin's chromogens would still remain an important one.

As has been pointed out, V. I. Palladin predicted the discovery of new "chromogens" or intermediary hydrogen carriers of a quinonoid nature. Convincing evidence has been obtained of recent years in confirmation of this prediction, which was based on a profound analysis of the respiratory process. Although the role of α-tocopherol (or, more precisely, of α-tocopherylquinone) in transport of electrons in the respiratory chain has so far been shown only for mitochondria of animal cells, it is probable that it also applies to plant cells. V. I. Palladin emphasized, as far back as 1915, the identity or close similarity of the basic respiratory mechanisms of plant and animal cells. The same applies to the respiratory role of the naphthoquinones of the vitamin K group. The exhaustive researches, involving the most advanced experimental techniques, leading to the identification and clarification of the role of quinones of the Q group (or coenzymes) in the respiratory process (1959) should also be mentioned. The brilliant work of Crane and his associates leaves no doubt as to the existence in all aerobic cells of a series of benzoquinone derivatives, which are important components of respiratory systems, in particular of the succinoxidase system. These intermediary catalysts are complex compounds of the type of 2, 3-dimethoxy-6-polyisoprenyl-6-methylbenzoquinone.



V. I. Palladin could not have foreseen the existence of such complex compounds. However, they afford striking confirmation of the correctness of his carefully developed theories on the mediatory role of quinones in hydrogen or electron transport from the substrate molecule to oxygen.

In reading the papers of V. I. Palladin, published over 50 years ago, one cannot but feel admiration for the profound scientific perspicacity of this research worker, for the breadth of his chemical and biological vision, and for the tenacity with which he upheld his views.

D. M. Mikhlin

ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN RUSSIAN BIO-SCIENCES LITERATURE

Abbreviation (Transliterated)	Significance
AMN SSSR	Academy of Medical Sciences, USSR
AN SSSR	Academy of Sciences, USSR
BIN	Biological Institute, Botanical Institute
FTI	Institute of Physiotherapy
GONTI	State United Sci-Tech Press
GOST	All Union State Standard
GRRRI	State Roentgenology, Radiology, and Cancer Institute
GTTI	State Technical and Theoretical Literature Press
GU	State University
I Kh N	Scientific Research Institute of Surgical Neuropathology
IL (IIL)	Foreign Literature Press
IONKh	Inst. Gen. and Inorganic Chemistry (N. S. Kurnakov)
IP	Soil Science Inst. (Acad. Sci. USSR)
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd.	Press
LEM	Laboratory for experimental morphogenesis
LENDVI	Leningrad Inst. of Dermatology and Venereology
LEO	Laboratory of Experimental Zoology
LIKhT	Leningrad Surgical Institute for Tuberculosis and Bone and Joint Diseases
LIPZ	Leningrad Inst. for Study of Occupational Diseases
LIPK	Leningrad Blood Transfusion Institute
Medgiz	State Medical Literature Press
MOPISh	Moscow Society of Apiculture and Sericulture
MVI	Moscow Veterinary Institute
MZdrav	Ministry of Health
MZI	Moscow Zootechnical Institute
LOKhO	Leningrad Society of Orthopedic Surgeons
NIIZ	Scientific Research Institute of Zoology
NINKhI	Scientific Research Institute of Neurosurgery
NIU	Scientific Institute for Fertilizers
NIUIF	Scientific Research Institute of Fertilizers and Insecticides
NIVI	Veterinary Scientific Research Institute
ONTI	United Sci. Tech. Press
OTI	Division of Technical Information
RBO	Russian Botanical Society
ROP	Russian Society of Pathologists
SANIIRI	Central Asia Scientific Research Institute of Irrigation
SANIISh	Central Asia Scientific Research Institute of Sericulture
TsNII	All-Union Central Scientific Research Institute
TsNTL	Central Scientific and Technical Laboratory
VASKhNIL	All-Union Academy of Agricultural Sciences
VIG	All-Union Institute of Helminthology
VIEM	All-Union Institute of Experimental Medicine
VIR	All-Union Institute of Plant Cultivation
VIUAA	All-Union Institute of Fertilizers, Soil Science, and Agricultural Engineering
VIZR	All-Union Institute of Medical and Pharmaceutical Herbs
VNIRO	All-Union Scientific Institute of Fishing and Oceanography
ZIN	Zoological Inst. (Acad. Sci. USSR)

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. - Publisher.

RUSSIAN JOURNALS FREQUENTLY CITED

[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiia	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiia	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Embriol.	Arkhiv Anatomii Gistologii i Embriologii	Archives of Anatomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiia	Biokhimiia	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiia Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Biull. Eksptl. Biol. i Med.	Biulleten Eksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Biull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Biulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Eksptl. Khirurg.	Eksperimentalnaia Khirurgiia	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiia i Toksikologiia	Pharmacology and Toxicology
Farmatsiia	Farmatsiia	Pharmacy
Fiziol. Rastenii	Fiziologiia Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenova Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariia	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiia Akademii Nauk SSSR, Seriia Biologicheskaia	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiia Tikhookeanskogo N. I. Instituta Rybnogo Khoziaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiia	Khirurgiia	Surgery
Klin. Med.	Klinicheskaia Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo(po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaia Parazitologiia i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaia Radiologiia	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiia	Mikrobiologiia	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhiat. i Psikhogig.	Nevropatologiia, Psikhiatriia i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediia, Travmatologiia i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriia	Pediatriia	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Endokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniia Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaia Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journals
Stomatologiia	Stomatologiia	Stomatology
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriia	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics

* LIBRARY OF CONGRESS TRANSLITERATION SYSTEM.

(continued)

(continued)

Abbreviation	Journal	Translation
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiia	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiia	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiia, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoiuz. Gidrobiol. Obshchestva	Trudy Vsesoiuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoiuz. Inst. Ekspti. Med.	Trudy Vsesoiuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiia	Urologiia	Urology
Uspekhi Biokhimiia	Uspekhi Biokhimiia	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiia	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriia Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universiteta, Seriia Biologii i Pochvovedeniia	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiia	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariia	Veterinariia	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirugii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniia	Voprosy Pitaniia	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaia Laboratoriia	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
Zhur. Nevropatol. i Psikhiat.	Zhurnal Nevropatologii i Psikhiatrii imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiia	Journal of General Biology
Zhur. Vysshei Nerv. Deiatel.	Zhurnal Vysshei Nervnoi Deiatelnosti imeni I. P. Pavlova	I. P. Pavlov Journal of Higher Nervous Activity
Zool. Zhur.	Zoologicheskii Zhurnal	Journal of Zoology

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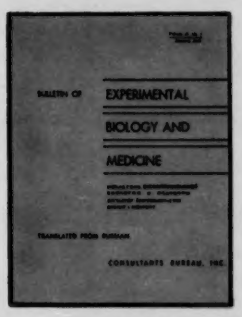

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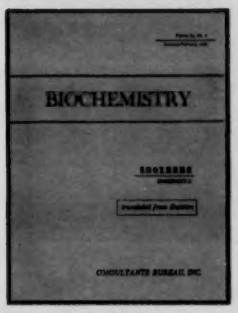

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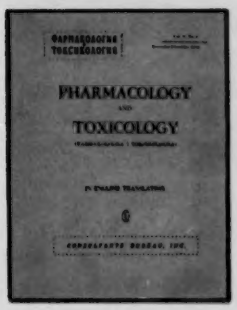

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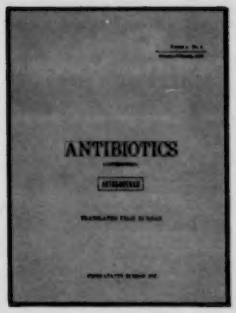

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